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PRINCIPAL INVESTIGATOR: Thomas M. Mack, M.D.

CONTRACTING ORGANIZATION: University of California
Los Angeles, California 90033

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6. AUTHOR(S)
Thomas M. Mack, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Southern California
Los Angeles, California 90033

E-Mail: tmack@hsc.usc.edu

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Activities included 1) acquisition of archived tissue blocks on breast cancer concordant twin pairs (205 MZ and 129 DZ) and discordant pairs (550 MZ); 2) immunohistochemistry to detect the level of expression of biomarkers (p53, HER-2/*neu*, ER, and PR) in the tumor tissue; 3) DNA sequencing of the p53 gene; 4) FISH to detect amplification of HER-2/*neu*; and 5) analysis of the association of environmental and genetic factors with the development of these markers. Blocks from 488 twins (254 in concordant pairs and 234 in discordant pairs) have been received. Based on 46 concordant pairs, intra-pair analyses showed that ER was the biomarker most likely to be identical within the pair (Kappa=0.43) and HER-2/*neu* was the least likely (Kappa=-0.07). The low agreement suggests that environmental factors are likely to play a role in the development of some of these tumor markers. DNA sequencing of the p53 gene has been completed for 67 cases, with 16 having at least one mutation. FISH has been completed on 81 cases. The FISH results were in high agreement with the HER-2/*neu* IHC results; however the p53 mutation results were not correlated with the p53 IHC results. Analysis of risk factors showed that late menarche and smoking were associated with the development on an ER+ or PR+ tumor; whereas OC use was protective.

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A. INTRODUCTION

Abnormalities relating to the p53 gene are one of the most commonly found genetic aberration in breast cancer tumors, and include overexpression of p53 protein, loss of heterozygosity at the p53 locus, and specific mutations in the p53 gene. However, it is unknown why some tumors have these changes and others do not. Further, little is known about what factors are involved in the interaction of oncogenes such as HER-2/neu with p53.

While investigators in previous studies have attempted to link p53 abnormalities to tumor histology, survival time, estrogen and progesterone receptor status, Her-2/neu, and, in some cases, risk factors for breast cancer, none has studied all of these factors within a large population of twins. These subjects offer great potential for distinguishing the role of predisposing genetic factors from environmental exposures. Specifically we will address the following issues in this study: 1) Are genetically similar tumors more likely to occur among identical twins than among fraternal twins? 2) Do environmental factors predispose to concordance or discordance of genetic abnormalities? 3) Do fraternal twins, concordant for environmental exposures, tend to be discordant for genetic abnormalities, suggesting that other predisposing genetic factors that can be identified? 4) Among identical twins discordant for disease, are specific environmental factors more related to tumors with a genetic abnormality than those without?

Three methods have been commonly used to detect p53 abnormalities: immunohistochemical methods of detecting overexpression of the mutant p53 protein, polymerase chain reaction (PCR) techniques for the detection and sequencing of specific p53 mutations, and Southern blots to detect loss of heterozygosity (LOH) at the p53 gene locus. Studies have indicated that 50-60% of breast tumors may have LOH in the 17p region; there may be overexpression of the p53 mutant protein in 27-54% of all breast tumors (3). Specific mutations in the p53 gene usually occur in the highly conserved exons 5-8 (4,5). Twenty-five percent have been shown to occur in codons 245, 248, 273, and 282 (6). From collaborative efforts of specific p53 mutations in more that 30 types of cancer it has been shown that different types of cancer evince different patterns of DNA base substitutions (7).

Rarely have all types of abnormalities been investigated within the same tumor tissue, but a few studies provide information on the correlations between them. Overexpression of the mutant p53 protein product has been seen in association with mutation of the p53 gene (8) but not invariably (9). LOH and overexpression of the p53 protein have been found to occur independently (9,10,11). The mechanism by which dysfunction in the p53 gene leads to malignant transformation is therefore unclear.

Under one hypothesis it would be necessary for both copies of the p53 gene to be inactivated by loss or mutation to prevent the transcription of the normal or 'wild-type' protein and hence prevent normal function of the gene. The failure by some investigators to demonstrate damage to or loss of both copies of the p53 gene suggests that additional steps or other mechanisms must

precede malignant transformation. For example, under a hypothesis of co-dominance, a stable mutant protein might bind to and inactivate any wild-type protein produced (12). Strong immunohistochemical staining for p53 in normal cells has been found in a mother and daughter with a family history of breast cancer (13). However, no p53 overexpression was found in fibroblasts from individuals from families with the Li-Fraumeni syndrome who had germline DNA mutations of the p53 gene (14). Thus another event (apart from damage to p53) sometimes may be necessary for expression of mutant protein, or only certain mutations in p53 may be related to overexpression of the mutant protein and subsequent malignant transformation.

Another mechanism by which the normal function of p53 gene may be interrupted is by nuclear exclusion (15). When p53 protein is found in the nucleus of cells, mutations in the gene are usually found, whereas when the protein is found in the cytoplasm, mutations are generally not found. If the protein is sequestered in the cytoplasm (by binding with heat shock proteins) then it may be unable to regulate nuclear division. Some studies have shown p53 protein to occur in the cytoplasm of lobular breast cancers (16).

When p53 mutations in germline tissue were found in members of Li-Fraumeni families (17), efforts to detect germline mutations in other high-risk families were intensified, largely without success (18, 19, 20). While these studies were based on small numbers of families: 5 (18) and 25 (19), or cases: 19 individuals with bilateral disease (20). This failure has led to the presumption that environmental factors or other genes may also determine the abnormalities in the p53 gene that lead to breast cancer (21). In any event, the inactivation or disabling of the p53 gene appears to be an important step in a large proportion of breast cancer cases, and studies have shown it to be an early step, present in *situ* tumors and maintained throughout all stages of tumor progression (8).

Since the etiology of breast cancer appears to be complex and heterogenous, other genes, especially oncogenes, may sometimes interact with p53 in the development and progression of breast cancer. HER-2/neu (or also referred to as c-erbB-2), located on the long arm of chromosome 17 (17q12-21.32) has been shown to occur in 20% of invasive breast cancer tumors and in 50% of all ductal carcinoma in situ (22). Studies that have examined the association of p53 with HER-2/neu have produced mixed results; at least four have found the two to be correlated (23, 24, 25, 26), while others have not (27, 28). Barbareschi et al. (26) suggest that p53 and HER-2/neu alterations may occur independently and at an early stage of tumor progression. Escape from hormonal control may be associated with HER-2/neu overexpression (which has been related to estrogen receptor negative tumors); while alterations in p53 may induce a high proliferation rate, leading to tumor progression and further opportunities for genetic damage.

The association of p53 abnormalities and HER-2/neu overexpression with estrogen and progesterone receptor status, histology, progression, and patient survival may provide insights into the mechanisms of tumor development and progression. While some studies have linked p53 overexpression to tumors with a more aggressive phenotype (28), it may be that LOH is

more critical to tumor progression than any specific mutation (11). Nuclear p53 expression has been associated with tumors of aggressive (ductal) as well as less aggressive (medullary) histology (16); however neither LOH nor specific mutation sequences were assessed. HER-2/neu is generally found in association with a poorer prognosis (29).

The relationship of p53 and HER-2/neu overexpression to environmental and other genetic risk factors has not been extensively studied. A higher proportion of tumors with p53 protein expression in familial than in sporadic cases has been reported (30). p53 has been associated with low levels of estrogen receptors (23, 26, 28) and late age at first full term pregnancy has been linked to the prevalence of estrogen receptors (McTiernan et al., 1986). An effect of breast-feeding on risk has been found to be dependent on expression of HER-2/neu (32).

To assess the interrelationships of tumor suppressor genes, oncogenes, specific mutations, loss of heterozygosity, and protein overexpression, it is essential that all factors be examined in the same material. This study presents the opportunity to study the several characteristics of breast cancer tumors in a large group of familial cases--concordant twin pairs--and relate these findings to genetic identity and to environmental risk factors. Secondly, a large number of disease discordant identical twin pairs offers the opportunity to further study association of environmental factors with specific genetic changes in breast cancer tumors.

B. BODY

Work done during the project has included the following:

1) Acquisition of Archived Tissue Blocks
    a) Contact with twins
    b) Correspondence with hospitals

2) Laboratory procedures
    a) Processing: Logging in of received blocks and slides in database and processing of tissue blocks to cut and store slides.
    b) Immunohistochemistry: p53, HER-2/*neu*, ER and PR
    c) DNA sequencing of the p53 gene from concordant pairs.
    d) FISH to detect amplification of HER-2/*neu* oncogene

3) Epidemiologic analyses
4) Results

1). Acquisition of Archived Tumor Blocks

a. Contact with Twins

Three groups of twins were contacted (concordant MZ and DZ twins and discordant MZ twins) and results are shown in Table 1. There were a total of 1,218 cases for whom we sought archived tissue blocks. Our procedures for contacting the twins were the same for each group. Beginning with those who were diagnosed after 1975 and for whom we had already obtained pathology reports, we sent a letter explaining the study, the informed consent, and a release form to each twin for her signature. If we determined that a twin was deceased, these forms were sent to her next of kin. If we did not receive a response from a twin after 4 weeks, we called the twin to be sure they received the forms and to answer any questions. Additional follow-up was performed as required. For those with diagnosis dates before 1975, we called the hospitals first to determine if the tissue blocks were still available, before initiating the correspondence with the twin. Of the 85 hospitals called, blocks were available for approximately 30%.

b. Correspondence with Hospitals

Once the signed informed consent and release forms were obtained from a twin, a letter was sent to the hospital along with the release form requesting the tissue blocks, including one that was most representative of the tumor and one that contained normal tissue, such as a lymph node. If the hospital's policies prohibited sending the blocks, we requested that 20 unstained slides be cut from each of the blocks specified, and sent to us. For hospitals not responding follow-up efforts were initiated.

2) Laboratory Procedures

a. **Processing**: Once the blocks (or slides) are received, they were transferred to Dr. Press's Laboratory in padded envelopes which had the Twin ID number, name of submitting hospital, and number of blocks and/or slides provided. This information was logged into a master data file. Variables in this file included information the characteristics of the tissue, number of blocks, number of nodes sampled, and patient information. One H&E slide is cut from each block submitted. Since numerous blocks were sent with some specimens, this enabled us to pick a block that was most representative of the tumor and one that was most representative of normal tissue. The 20 unstained slides were then cut from the chosen blocks and were then coated with paraffin so that antigenicity was not lost during storage. After this process was completed, the blocks were sent back to the hospitals.

b. Immunohistochemistry: p53, HER-2/neu, ER and PR

When a specimen was selected to be stained, two slides per analysis were taken. One was for the antibody of interest and the other was used as a negative control. A positive control was used for every antibody on each day's run. The antibodies were scored on the basis of intensity of staining. HER-2/neu, being a membrane protein, was scored as low (+), over-expressed (++), or highly over-expressed (+++). A tumor was considered to be positive if the staining was either over-expressed or highly overexpressed. P53, ER, and PR, which are nuclear proteins, are scored both by staining intensity and by percentage of cells with that particular intensity, i.e. (27%,

+++), (33%, ++), (10%, +). For the analyses, we considered a tumor to be positive if more than 10% of the cells were positive.
The laboratory procedures followed for each method are included in the Appendix.

c. DNA sequencing of the p53 gene: Our original plan was to do SSCP and then sequence only the portion of the gene with a mutation indicated by SSCP. However, in order to avoid the problems with lack of sensitivity inherent in the SSCP process, and since the technology for sequencing the gene had advanced since the grant was written we instead sequenced the entire gene directly, and eliminating the SSCP process. DNA yield is lower in the paraffinized tissue than in frozen tissue and the sequencing gives weaker peaks. This has required us to request more material for some cases. The laboratory procedures used are included in the appendix.

d. Fluorescence In Situ Hybridization (FISH). FISH is a method using DNA probes to localize genes in cytogenetic chromosomal spreads, in cytologic preparations of whole cells, or in histologic sections of tissue. Hybridization of the probes to their complementary genetic elements in cells is recognized by visualizing a fluorescence signal in cell nuclei with a fluorescence microscope. Initially, in our preliminary studies we used a series of 10 established breast cancer cell lines to confirm that each cell line, known to be amplified by Southern hybridization data, was amplified by FISH. The probe for HER-2/*neu* hybridized with both metaphase and interphase DNA to yield signals that are proportional with the known gene amplification level of the cell lines. The laboratory procedures followed are included in the appendix.

3). Epidemiologic Analyses
The laboratory findings were linked to environmental factors obtained from, a detailed questionnaire that was sent to all of the breast cancer twins and co-twins, which covered reproductive, developmental, and putative environmental breast cancer risk factors. Because the questionnaire included many questions about the co-twin, pairs in which only one twin responded were able to be used in the analysis. We have addressed the following objectives:
a) Within the concordant pairs in each zygosity group, determine if discordance in genetic abnormalities in tumor tissue is associated with discordance in environmental risk factors.
b) Within the MZ discordant pairs, determine if discordance in breast cancer is associated with discordance in environmental risk factors.
c) Within the MZ discordant pairs, determine if the relationship between environmental risk factors and breast cancer is the same for tumors with and without somatic abnormalities.

4). Results
a. Acquisition of archived tissue blocks
We have obtained blocks or unstained slides from 488 or 40.1% of the cases (Table 1). Reasons that blocks were not obtained were the following: almost 44% of the blocks were unavailable, 5% of the twins refused permission, 8% of the twins were lost and we did not know the hospital where they had had their surgery, and at the close of the study 3% were still pending at various hospitals. It required extensive follow-up efforts to obtain the blocks from the hospitals and

these remaining were the most difficult to obtain, after repeated attempts. The year of diagnosis was a major factor in determining the availability of the blocks because some hospitals keep blocks for only a certain length of time and, as the number of years increased between the diagnosis and our attempts to obtain the blocks, the less successful we were. We obtained 21% of those diagnosed before 1975 and 56% of those diagnosed between 1990-93 (Table 2). We were more successful at obtaining blocks from cases who were still alive at the time of the study (43% obtained) vs. those were deceased (33% obtained). Some differences were evident by category of twin pair as described below:

1) **MZ concordant twins:** 205 pairs of identical female twins, concordant for breast cancer, were initially selected to obtain archived tissue blocks. From these 410 cases we obtained tissue blocks from 187 or 45.6%. We were the most successful with this group in comparison to the DZ concordant and MZ discordant pairs. We had the highest acquistion rates for these pairs among all years of diagnosis, vital status categories, and particularly among those diagnosed at 50+, where we obtained blocks from 56%.
2) **DZ concordant twins:** We initiated efforts to obtain consent and release forms from 129 DZ concordant pairs, by sending letters first to twins who were diagnosed after 1975 and known be alive at last contact. We also sent letters to the next of kin to those who were known to deceased. We obtained blocks from 67 or 26.0% of these 258 cases. We also were more successful in obtaining blocks from the cases diagnosed over 50.
3) **MZ discordant twins:** We also selected 550 MZ discordant pairs who met the following criteria: a) they were diagnosed after 1975 and we had obtained their pathology report, and b) they had completed the epidemiologic questionnaire that was sent to all females pairs of twins with at least one member with breast cancer who participated in the International Twin Study Registry. We received blocks from 234 (42.5%) of these cases. There was no difference in our success rate in this group by age at diagnosis.

Among concordant pairs for whom we have received blocks, we have 68 pairs with blocks received from both twins (58 MZ and 10 DZ) and 118 additional pairs with blocks received from one twin (71 MZ and 47 DZ). Thus, in total we have received blocks from at least one twin for 186 concordant pairs (129 MZ and 57 DZ) which represent 62.6% of the MZ concordant pairs on whom we sought tissue and 44.2% of the DZ concordant pairs. In addition we have received blocks from 234 of the 550 discordant MZ pairs or from 42.5% of these pairs.

We do not have tumor related laboratory results on all of these pairs, however. For some pairs, only benign or normal tissue was obtained and for others there were problems with the quality of the tissue and the laboratory tests were not able to be performed. In addition, a small number of blocks were obtained after the laboratory component of the study was completed. In total we have laboratory results on 393/488 cases with blocks (80.5%). The proportions with laboratory results available are shown in Table 2 by subgroup. Overall laboratory results are available on 32.2% of the total cases, 37% of the cases from MZ concordant pairs, 20% of the cases from the DZ concordant pairs and 35% of the cases in MZ discordant pairs.

Among concordant pairs for whom we have laboratory results, we have 46 pairs with results from both twins (38 MZ and 8 DZ) and 111 additional pairs with results from one twin (76 MZ and 35 DZ). Thus, in total we have results from at least one twin for 157 concordant pairs (114 MZ and 43 DZ) which represent 55.3% of the MZ concordant pairs on whom we sought tissue and from 33.3% of the DZ concordant pairs.

Table 1: Final status of twin participation and acquisition of blocks/slides by category of pair

	Total Pairs		Category of Pair					
			MZ Concordant		DZ Concordant		MZ Discordant	
Status	N	%	N	%	N	%	N	%
Total pairs	884		205		129		550	
Total cases	1218	100.0	410	100.0	258	100.0	550	100.0
Blocks/Slides Obtained	488	40.1	187	45.6	67	26.0	234	42.5
Blocks/Slides N/A	541	44.4	176	42.9	125	48.4	240	43.6
Twin Refused	56	4.6	19	4.6	11	4.3	26	4.7
Patient Lost	100	8.2	23	5.6	51	19.8	26	4.7
Hospital Not Responsive	33	2.7	5	1.2	4	1.6	24	4.4

Table 2: Characteristics of Cases by Percent with Tissue Obtained and Percent with Laboratory Results Completed*

Selected Characteristics	Total Pairs		Category of Pair					
			MZ Concordant		DZ Concordant		MZ Discordant	
	Tissue	Lab	Tissue	Lab	Tissue	Lab	Tissue	Lab
All Cases	40.1	32.2	44.4	37.0	26.0	19.6	42.5	34.6
Year of Dx								
<1975	21.1	16.4	23.8	19.6	15.8	9.8	**	**
1975-79	28.2	19.9	40.6	26.1	25.6	20.5	21.3	15.7
1980-84	40.4	31.6	44.4	38.9	28.6	14.3	40.6	31.2
1985-89	49.8	41.6	65.1	55.3	34.0	28.0	47.8	39.6
1990-93	56.5	44.6	66.7	55.6	42.1	31.6	56.5	43.5
Age of Dx								
<50	35.2	27.6	33.2	26.7	22.3	17.9	42.9	32.9
50+	44.8	36.9	55.7	46.2	31.1	23.0	43.0	36.4
Vital Status								
Deceased	32.9	27.4	37.7	34.4	20.2	14.1	37.4	30.6
Alive	43.2	34.4	48.8	38.1	29.2	23.0	44.9	36.5

*For some cases for whom some tissue was obtained, no tumor tissue was obtained (although normal tissue may have been obtained) or the tissue obtained was unsuitable for processing.
**Cases in this group were selected from those diagnosed after 1975.

b. Immunohistochemistry for p53, HER-2/*neu*, ER, and PR

1) Percent positive

The percent of tumors that stained positive for each of the biomarkers is shown in Table 3. For all cases studied, the percent positive was 58.6% for ER, 56.3% for PR, 24.8% for p53, and 18.9% for HER-2/*neu* . Some differences by age at diagnosis were evident as ER, PR, and HER-2/*neu* were higher among those cases diagnosed at 50 or older than among younger cases; whereas little variation by age was seen for p53. By pair type, the percent positive for ER and PR were lower among the MZ discordant pairs than the concordant pairs, and this difference was especially true among the older cases, where, for example the ER % positive was 52.2% for the discordant pairs vs. 79.6% and 86.7% among the MZ and DZ concordant pairs, respectively.

Table 3: Immunohistochemistry Results for Individual Cases by Pair type

Percent Positive* for	Total Cases	Pair type		
		MZ Concordant	DZ Concordant	MZ Discordant
(N)	(391)	(151)	(50)	(190)
All ages of dx				
ER	58.6	68.7	71.4	47.4
PR	56.3	60.9	62.0	51.0
P53	24.8	27.8	22.0	23.2
HER-2/neu (M+H)	18.9	24.5	12.0	16.3
Age of dx <50	(148)	(49)	(20)	(79)
ER	43.5	46.9	47.4	40.5
PR	46.6	49.0	55.0	43.0
P53	26.5	32.6	15.8	25.3
HER-2/neu (M+H)	15.5	16.3	10.0	16.5
Age of dx 50+	(243)	(102)	(30)	(111)
ER	67.8	79.6	86.7	52.2
PR	62.1	66.7	66.7	56.8
P53	23.8	25.5	25.8	21.6
HER-2/neu (M+H)	21.0	28.4	13.3	16.2

*Positivity for ER, PR, and p53 defined as expression in more than 10% of cells; positivity for HER-2/neu defined as both medium or high expression (M+H).

2) Correlation of positivity of different biomarkers within tumors

The correlation of positivity for the four different biomarkers within tumors by pair type and age at diagnosis is shown in Table 4. As expected, ER and PR were highly significantly positively correlated with each other in all subgroups. These correlations were the strongest among those diagnosed <50 years of age. ER and P53 were consistently negatively correlated with each other, although no specific association with age at diagnosis was seen. PR and P53 were also generally negatively correlated and this association appeared strongest among the younger cases. ER and HER-2/*neu,* as well as PR and HER-2/*neu,* also tended to be negatively correlated with each other, especially among older cases.. P53 and HER-2/*neu* tended to be positively correlated with each other overall, but this positive association was only evident among the older cases.

Table 4: Correlation coefficients between tumor biomarkers within same tumor by age group of diagnosis and Pair type

Age group And Pair Type	Biomarker Correlations					
	ER-PR	ER-P53	ER-HER-2	PR-P53	PR-HER-2	P53-HER-2
All Pair types						
All ages (N=391)	+0.59*	-0.18*	-0.10*	-0.13*	-0.14*	+0.12*
Age dx <50 (N=148)	+0.70*	-0.22*	+0.12	-0.29*	-0.02	-0.05
Age dx 50+ (N=243)	+0.50*	-0.15*	-0.26*	-0.03	-0.22*	+0.22*
MZ Concordant All (N=151)	+0.52*	-0.23*	-0.02	-0.02	+0.02	+0.02
Age of Dx <50 (N=49)	+0.55*	-0.13	+0.37*	-0.25	+0.24	-0.08
Age of Dx 50+(N=102)	+0.48*	-0.28*	-0.30*	+0.13	-0.11	+0.08
DZ Concordant All (N=50)	+0.70*	-0.26	-0.45*	-0.14	-0.47*	+0.12
Age of Dx <50 (N=20)	+0.90*	-0.15	-0.32	-0.22	-0.37	-0.15
Age of Dx 50+(N=30)	+0.55*	-0.48*	-0.71*	-0.11	-0.55*	+0.25*
MZ Discordant All (N=190)	+0.61*	-0.15*	-0.13	-0.24*	-0.22*	+0.20*
Age of Dx <50 (N=79)	+0.74*	-0.30*	+0.05	-0.33*	-0.11	-0.02
Age of Dx 50+(N=111)	+0.51*	-0.02	-0.26*	-0.16	-0.31*	+0.36*

*p<.05

3) Agreement of Biomarker Expression Between Cases of Concordant Pairs

From the 191 twins from the concordant pairs with immunohistochemistry results, there were 46 pairs (38 MZ and 8 DZ) with results available from both members of the pair. We examined the percentage of these pairs that had the same expression (either positive or negative) of each of the biomarkers and compared that to what would have been expected, given the marginal distributions. Kappa, a measure of agreement after taking chance agreement into account, was also calculated. As shown in Table 5, with all ages of diagnosis combined, the agreement of ER expression between the pair members was the only significant finding. However, significant results were seen for all biomarkers for those with an age of diagnosis under 50. ER, PR, and p53 were all in higher agreement than would have been expected by chance, but HER-2/*neu* expression was actually less likely to be the same than would have been expected. Among those diagnosed at 50 or older, there was some increased agreement for ER, but no association for any of the other markers.

Table 5: Measures of Agreement Between Biomarkers from Members of Concordant Pairs with Tissue Analyzed from Both Twins

Age at diagnosis	Percent Agree	Percent Expected*	Kappa
All ages at dx (N=46)			
ER	74.4	55.3	0.43**
PR	62.2	49.2	0.26
P53	68.2	58.3	0.24
HER-2/*neu* (M+H)	60.9	63.7	-0.07
Age at dx <50 (N=14)			
ER	76.9	50.8	0.53**
PR	84.6	49.2	0.70**
P53	83.3	51.5	0.66**
HER-2/*neu* (M+H)	64.3	70.2	-0.21**
Age at dx 50+ (N=32)			
ER	70.0	64.1	0.26
PR	53.1	50.0	0.06
P53	62.5	62.5	0.00
HER-2/*neu* (M+H)	59.4	60.6	-0.03

*expectation of agreement based on marginal distributions
**p<.05

c. DNA Sequencing of the p53 gene

Concordant pairs were selected for sequencing of the p53 gene. Exons 4-9 were sequenced. At the end of the fourth year of the project, 50 cases were sequenced and alterations were detected in 25. However, this was a preliminary result obtained by sequencing only one DNA strand from each exon in the sense direction. Since the DNA products are double stranded, the initial findings needed confirmation by DNA sequence analysis of the opposite strand in the anti-sense direction. This was performed during the subsequent year and the analysis of the opposite strand confirmed alterations in 10 of the 25 cases. In addition, 17 additional cases were sequenced in both the sense and anti-sense directions and 6 p53 alterations were identified in these cases. Thus, in total, from 67 cases studied, 16 were found to have either a mutation or polymorphism in the p53 gene.

These 67 cases included 26 pairs with results from both members (52 cases) and an additional 15 pairs with results from one member. The specific mutations or polymorphisms found in the 16 cases with confirmed alterations are described in Table 6. There was one concordant pair (#7550) with identical silent polymorphisms; however, in general there was a lack of correlation in presence of a p53 mutation or polymorphism between members of the 26 pairs with results for both members, when including all alterations as well as when restricting the analysis to only mutations with functional significance (Table 7). The immunohistochemistry results for ER and

PR were significantly correlated between members of these pairs, as was shown previously with the complete data set.

Table 6

TWIN Results
p53 Mutations exons 4-9

Mutations						
Case No.	Exon	Codon	nucleotide	DNA Sequence	Amino Acid Sequence	Mutation Type
2188B	4A	71	12137	GGG->AGG	Gly->Arg	Missense
4601A	4A	75	12148	GGA--> GAA	Gly--> Glu	Missense
7191A	4A	89	12190	GGG--> GAG	Gly--> Glu	Missense
7691B	4A	68	12126	GAG--> TAG	Glu--> stop	Nonsense
11754A	4A	75	12148	GGA--> GAA	Gly--> Glu	Missense
7755A	5	126	13055	TAC->GAC	Tyr->Asp	Missense
9866B	5	175	13203	CGC->CAC	Arg->His	Missense
6322A	6	192	13334	CAG--> TAG	Gln--> stop	Nonsense
11772B	7	240	14047	AGT->AGG	Ser->Ser	Silent
	7	260	14106	TCC->TAC	Ser->Tyr	Missense
1523B	9	325	14735	GGA->GAA	Gly->Glu	Missense
1529A	intron 9		14766	T->C		unknown significance
2497A	intron 9		14765	T->C		unknown significance
Polymorphic Mutations						
6322A	4A	72	12139	GCG--> GGG	Ala--> Gly	Non-silent Polymorphism
6310A	6	213	13399	CGA--> CGG	Arg--> Arg	Silent polymorphism
7550A	6	213	13399	CGA--> CGG	Arg--> Arg	Silent polymorphism
7550B	6	213	13399	CGA--> CGG	Arg--> Arg	Silent polymorphism
9866A	6	213	13399	CGA--> CGG	Arg--> Arg	Silent polymorphism

Table 7: Correlation between p53 DNA sequencing results and immunohistochemistry results for other biomarkers between members of 26 concordant pairs.

Biomarker	Percent Agree	Expected agreement	Kappa
P53 mutation/polymorphism	57.7	57.7	-0.01
P53 mutations only	61.5	69.2	-0.24
P53 (IHC)	70.8	56.8	0.32
ER	81.8	53.5	0.61*
PR	66.7	47.2	0.37*
HER-2/*neu*	58.3	61.9	-0.10

*p<.05

Using all 67 cases with p53 sequencing results, we also examined the correlation between the presence of a p53 mutation/polymorphism or a p53 mutation only with the p53 immunohistochemistry results and with the other IHC results within the same tumors. As shown in Table 8, there was a lack of correlation between the p53 sequence results (whether or not only mutations were included) and the p53 IHC results. Among the 65 individuals with both p53 sequence and p53 IHC results, 33 were negative for both, 4 were positive for both, 12 had a p53 mutation/polymorphism but were p53 negative (IHC), and 16 were positive for IHC but negative for the sequence result. The p53 DNA results were negatively correlated with ER positivity, however. There was also a positive, though non-significant association between the p53 DNA results and HER-2/*neu* positivity, while there was no association between the p53 IHC results and HER-2/*neu*.

Table 8: Correlation coefficients between p53 sequence results (including all alterations and mutations only) and IHC tumor biomarkers within same tumor for 67 cases from concordant pairs.

	Biomarker Correlations					
	P53 DNA (all)	P53 DNA (mut)	P53 IHC	ER	PR	HER-2/*neu*
P53 DNA (mutation/polymorphisms)	1.00	1.00				
P53 IHC	-0.07	+0.05	1.00			
ER	-0.36*	-0.47*	-0.32*	1.00		
PR	-0.03	-0.14	-0.10	+0.60*	1.00	
HER-2/*neu*	+0.20	+0.04	-0.05	-0.20	-0.21	1.00

*p<.05

d. FISH (Fluorescence In-Situ Hybridization)

Two methods were used to do the FISH analyses using tumor samples from twins from concordant pairs. In Year 4 of the project the ONCOR, Inc. assay (described in Appendix) was performed on 100 cases, with results successfully obtained from 96. Values of 4.0 and higher were defined as being positive, and the values ranged from 1 to 23.8. Based on the 4.0 cut point, 29/96 or 30.2% of the samples were positive for amplification.

In Year 5 of the project, an alternative method of measuring gene amplification was adopted in Dr. Press's Laboratory. The Vysis, Inc. method (described in Appendix) measures amplification of HER-2/*neu* as well as the number of Chromosome 17 copies. The ratio of the number of HER-2/*neu* copies to the number of Chromosome 17 copies is used to determine amplification and values of 2.0 or higher are considered to be positive for amplification. This test is more specific, yielding very few false positives. From 81 samples tested, 7 or 8.6% had ratios of 2.0 or higher. There were 73 samples with results from both methods of FISH and there was agreement between the two methods for 56 case or 76.7%. However Kappa was a low 0.28 (.06-

.50) and, among the 17 cases of with disagreement, 16 were positive by the ONCOR method and negative by the Vysis method. These differences are somewhat puzzling and are under further investigation. For 6 of the disagreements, the positive ONCOR value was between 4.4-4.6, which was just above the cut point (of 4.0).

The results from both methods of performing FISH were compared to the IHC results for HER-2/*neu*. Of the 96 cases with FISH performed by the ONCOR method, 82.3% were in agreement with the IHC results and Kappa was 55.8 (0.37-0.74). There were 6 cases that were positive by IHC and negative by FISH and 11 cases that were negative by IHC and positive by FISH. Among these cases 25.0% were positive by IHC vs. 30.2% by FISH.

In comparison, among the 81 cases with FISH performed by the Vysis method, 8.6% were positive vs. 24.7% with IHC. There was agreement between the two methods for 66 or 81.5% and Kappa was 0.36 (0.13-0.60). Among the 15 disagreements, 14 were positive by IHC and negative by FISH.

In conclusion, the Vysis method produced a substantially lower positivity rate for HER-2/*neu* amplification in comparison to the ONCOR and IHC results. Agreement between IHC and FISH was higher for the ONCOR method. However the Vysis method was likely to produce fewer false positives. These results, especially the Vysis results, should not be considered to be finalized.

e. Epidemiological Analyses: Association with Risk Factors

To study the possible role of environmental factors on the development of these biomarkers, we utilized risk factor information that was obtained from the twins when they first entered the Registry. Two types of analyses were conducted. First, using only cases, separate logistic regression models were utilized with each of the biomarkers as the dependent variable (ER, PR, p53, and HER-2/*neu*) and selected risk factors (including age at menarche, nulliparity, parity, OC use, current smoking, and lactation) as the independent variables to determine the association of these risk factors with a positive immunohistochemistry result. These analyses were conducted separately for all cases, all cases by age of diagnosis (<50, 50+), cases from concordant pairs, and cases from discordant pairs. Secondly, using only the discordant pairs, conditional logistic regression models were constructed to examine the association of selected risk factors with the risk of IHC + and – breast cancer for each of the markers studied (i.e. ER, PR, p53, and HER-2/*neu*).

Table 9 shows the adjusted odds ratios for the association of selected risk factors with a positive IHC result, based only on cases. Although Table 9 shows the results for all cases, similar results were found when stratified by age group and by cases from concordant and discordant pairs. An older age at menarche was associated with an increased risk of having an ER+ or PR+ tumor, but not with a p53 or HER-2/*neu* positive tumor. Parity appeared to have little association with any of the biomarkers. OC use was negatively associated with ER or PR positivity but had no

relationship to p53 or HER-2/*neu* positivity. Smoking was associated with ER and PR positivity. Finally lactation was not strongly associated with any of the biomarkers, although it had the strongest negative association with p53 positivity.

Table 9: Adjusted Odds Ratios and 95% C.L. Measuring the Association of Selected Risk Factors and IHC positivity for ER, PR, p53, and HER-2/*neu*.: Cases only

Selected Risk Factor Model	ER+	PR+	P53+	HER-2/*neu*+
All cases				
Menarche <12	1.2 (0.6-2.3)	0.8 (0.4-1.6)	0.8 (0.3-1.7)	1.0 (0.4-2.3)
Menarche 12	1.0 (ref.)	1.0 (ref.)	1.0 (ref.)	1.0 (ref.)
Menarche>12	2.1 (1.2-3.5)	2.3 (1.4-4.0)	0.8 (0.4-1.8)	0.8 (0.4-1.6)
Nulliparity	0.8 (0.4-1.6)	1.1 (0.5-2.2)	0.8 (0.4-1.8)	0.8 (0.3-2.0)
Parity 1-2	1.0 (ref.)	1.0 (ref.)	1.0 (ref.)	1.0 (ref.)
Parity 3+	1.0 (0.6-1.7)	1.3 (0.8-2.1)	1.2 (0.7-2.1)	1.1 (0.6-2.0)
OC use (ever)	0.4 (0.3-0.7)	0.5 (0.3-0.8)	1.0 (0.6-1.7)	1.0 (0.5-1.7)
Current smoker	1.7 (1.0-3.0)	2.4 (1.4-4.1)	0.8 (0.4-1.4)	0.9 (0.4-1.6)
Nursed 1+ children	1.0 (0.6-1.8)	0.8 (0.5-1.5)	0.6 (0.3-1.2)	1.3 (0.7-2.6)

Table 10 shows a standard case-control analysis for the discordant pairs, stratified by IHC status of the case's tumor for each of the biomarkers studied. With regard to ER and PR, an increased risk was seen for smoking and the IHC positive tumors, but not for the IHC negative tumors. This is a similar finding to the cases only analysis presented above where smoking was associated with the positive IHC result for ER and PR. For ER there was also a suggestion of heterogeneity by IHC status for the effect of age at menarche, with early menarche associated with increased risk of the ER- tumor but not of the ER+ tumor. For p53 and HER-2/*neu* current smoking was associated with an increased risk of the IHC negative tumor, but not for the IHC positive tumor. Also, for both of these biomarkers, nursing appeared to be protective for risk of the IHC+ tumor. OC use was associated with a significantly reduced risk of the HER-2/*neu* positive tumor.

Table 10: Adjusted odds ratios for selected breast cancer risk factors stratified by IHC results for each biomarker: Discordant MZ pairs.

Selected Risk Factor Model	Adjusted OR's from Models Stratified by IHC status of Case's Tumor							
	ER+	ER-	PR+	PR-	P53+	P53-	HER-2+	HER-2-
Number of pairs	87	94	94	87	42	139	31	150
Menarche <12	0.8	1.5	1.3	2.3				
Menarche 12 (ref.)	1.0	1.0	1.0	1.0				
Menarche>12	0.7	0.5	0.8	0.6				
(Or First Menarche)	(0.7)	(2.0*)	(1.2)	(1.4)	(1.6)	(1.2)	(1.6)	(1.2)
Nulliparity	0.5	0.8	0.7	0.5	1.1	0.8	Nc	1.0
Parity 1-2 (ref.)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Parity 3+	1.0	1.1	1.0	1.0	1.2	1.1	1.1	1.1
OC use (ever)	0.6	0.8	0.9	0.8	1.0	0.8	0.2*	1.2
Current smoker	2.5	0.8	2.8*	0.5	0.7	1.6	0.8	1.5
Nursed 1+ children	0.8	0.7	0.6	1.0	0.4	1.0	0.4	0.9

*p<.05

C. CONCLUSIONS

Key Accomplishments:

1) Tissue was able to be obtained from 40% of the cases and success was directly related to the year of diagnosis.
2) Immunohistochemistry was successfully performed on the paraffinized tissue. ER and PR were highly positively correlated within the same tumor tissue and were negatively correlated with p53 and HER-2/*neu* positivity. Between cases from concordant pairs, ER was the only biomarker to be significantly correlated overall; however the relationship was strongest among pairs with the first case diagnosed before age 50. In this subset, PR and p53 were also significantly correlated.
3) Except for one pair, we found no correlation in specific p53 mutations/polymorphisms between members of concordant pairs or in the occurrence of any mutation or polymorphism. Within these tumors p53 mutations/polymorphisms were not correlated with p53 IHC results.
4) Two methods of measuring gene amplification by FISH produced differing results, with the Vysis method yielding a lower rate of positivity than the ONCOR method. In comparison to the IHC results, higher levels of agreement beyond chance were seen for the ONCOR method.
5) Epidemiologic risk factor analyses showed that ER and PR positive tumors were more associated with a later age at menarche and with smoking and were less likely to occur after

OC use. There was some suggestion that p53 + and HER-2/neu + tumors were less likely to develop if the women had nursed at least one child. OC use may also help prevent HER-2/neu positive tumors.

Reportable Outcomes

1) Establishment of Resource of Heritable Breast Cancer Specimens

2) Presentations

Hamilton, A., Mack, T., and Press, M. "P53 and HER-2/*neu* Alterations in Breast Cancer Concordant Twins." Platform presentation and abstract. Era of Hope Meeting, Department of Defense Breast Cancer Research Program Meeting, Oct. 31-Nov. 4, 1997. Proceedings. Vol. 1, p. 163.

3) New grants obtained as a result of the resource of heritable breast specimens developed as a result of this project.

Ann Hamilton, P.I., 1999-00. Breast Cancer Genes in Very High Risk Women. California Breast Cancer Research Program. 5IB-0116. $131,197.

Ann Hamilton, P.I., 2000-03. Breast Cancer Genes in High Risk Women. Department of Defense Breast Cancer Research Program. DAMD17-00-1-0431, $366,970

4) Completed Manuscripts

Hamilton, A., Mack, T., and Press, M. 2000. Familiality and the Determinants of Breast Cancer.

5) Manuscripts in Preparation

Determinants of Success in the Collection of Archived Tissue

Breast Cancer Tumors Markers and Disease Heritability

Heritability of Breast Cancer Tumor Markers

P53 mutations and Protein Overexpression

HER-2/*neu* Oncogene Amplification and Protein Overexpression

Breast Cancer Risk Factors and Tumor Markers

E. Personnel Supported

Key Personnel

Thomas Mack, M.D., P.I.
Ann Hamilton, Ph.D. Co-P.I.
Michael Press, M.D., Ph.D. Co-P.I.

Office Staff (Assisted with block acquisition effort)
Ruby Sidhu (Project Assistant)
Gayle Alis (Research Assistant)
Laura Collins (Project Assistant)
Ashley Gallagher (Project Assistant)

Laboratory Personnel
Karen Petrosyan (Research Associate)
Ivonne Villalobos (Project Specialist)
Judy Udove (Research Associate)
Lin-Song Hsieh (Research Technician)
Jinha Park (Graduate Student)
Lynn Rusimovic (Data Manager)
Armine Arakelyan (Research Technician)
Ron Chai (Data Manager)
Dorothy Hong (Research Technician)
Brandon Li (Research Associate)
Ning Niu (Reseach Technician)
Jian Yuan Zhou (Research Associate)
Monica Barron (Research Technician)
Hasmik Toumaian (Data Manager)
Isabel Schmitt (Research Technician)
Allen Chudzinski (Research Technician)

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Appendix
Laboratory Procedures

'The Estrogen Receptor and Progesterone Receptor: Immunohistological Staining Protocol for Paraffin-Embedded Sections'

'Proto-Oncogene HER-2/*neu* Immunohistochemical Staining Protocol in Paraffin and Frozen Sections'

'P53 Tumor Suppressor Immunohistochemical Staining Protocol for Paraffinized Tissue'

'HER-2/*neu* Proto-Oncogene Fluorescence *In Situ* Hybridization (FISH) Protocol (Oncor, Inc.) in Tissue Sections'

'HER-2/*neu* Proto-Oncogene Fluorescence *In Situ* Hybridization (FISH) Protocol (Vysis, Inc.) in Tissue Sections'

'DNA Sequencing Protocol'

The Estrogen Receptor and Progesterone Receptor Immunohistochemical Staining Protocol for Paraffin-Embedded Sections

I. TIME-LINE OF PROCEDURE USE IN PRESS CONSULTATION LABORATORY.

Adopted: May 30,1990

Review Policy: Annually since procedure adopted by Dr. Michael Press.

Dates of Review for Last Two Years: January 30,1996, January 27, 1997, January 26, 1998 and January 22, 1999.

Changes to Procedure Since Adoption: 1. Estrogen Receptor staining changed from Abbott kit H222 Ab with an ABC technique to 1D5 Ab using an antigen retrieval technique IVC2a. 2. Progesterone Receptor staining did not change and is listed here under IVC2b.

Date of Change to Procedure: September 14, 1995.

Most Recent Review of Procedure: Janury 29, 2000 by Dr. Michael Press.

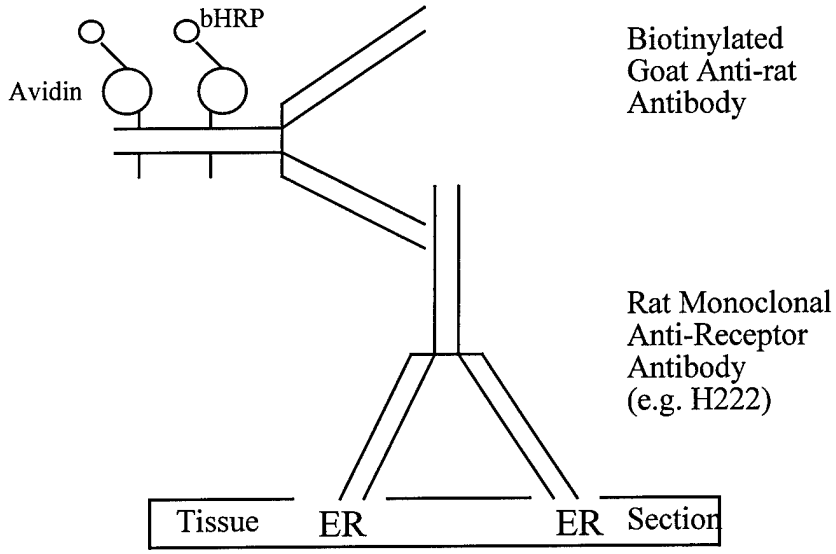
II. PRINCIPLE

A. REACTION

Rat monoclonal antibodies to the human estrogen receptor (ER) and human progesterone receptor (PR) are used with immunohistochemical techniques to demonstrate ER and PR proteins in tissue sections. Antibody bound to this antigen in tissue sections is visualized by use of antibody to the primary antibody and a chromogen that can be visualized microscopically after an enzymatic reaction.

The specificity of these antibodies for ER and PR has been demonstrated with biochemical assays and western immunoblot analyses (1-5). That these antibodies are also specific for their receptors in the immunocytochemical technique has also been confirmed with competition studies for both ER and PR using purified receptor protein (3-6).

The binding of monoclonal receptor antibody to its antigenic site in the tissue section is visualized by the use of a biotinylated second antibody, a goat anti-rat IgG antibody, to localize the sites where rat monoclonal receptor antibody is bound (7,8). This second biotinylated antibody is identified by incubation with avidin-biotin-peroxidase and subsequent incubation with the chromogen diaminobenzidine (7,8). The diaminobenzidine reaction product marks the location of the immunoprecipitates in the tissue section and is visualized microscopically.



B. CLINICAL

The ER and PR status of breast tumors has been shown to correlate with prognosis and clinical endocrine response to therapy (9). Although this correlation was first demonstrated with biochemical assays for ER and PR, subsequent work has shown a similar correlation for ER and PR determined with immunohistochemical assays (9). Most studies have demonstrated that clinical endocrine response is predicted with greater accuracy in immunohistochemical assays than in biochemical assays of ER (9, 10). Immunohistochemical assay has the added advantage that receptor content can be determined in smaller samples than the conventional biochemical assay requires (250-500 mg of frozen tissue). With improved diagnostic procedures permitting early identification of small tumors this is becoming a progressively more important issue. Although frozen tissue is the ideal sample, the immunohistochemical assay can be performed in paraffin-embedded tissue while this is not feasible for the biochemical assay.

III. SPECIMEN

A. SPECIMEN TYPE

Paraffin blocks or pre-cut unstained paraffin sections on slides.

B. HANDLING CONDITIONS

1. If the specimen is **paraffin-embedded** tissue block(s), open the container and check the number on the block(s) to confirm that it matches the number indicated by the referring institution. If it does not match, call the hospital and note this in our records.

2. The person opening and inspecting the referred tissue should sign his/her name or initials to our log book for later reference purposes.

4. Label the paraffin block with our consultation number as well as the paper from the referring hospital.

5. Copy all paperwork from the referring institution and keep it separately; log-in the patient name; our consultation number; hospital name address; date the specimen arrived; test(s) requested; and any special requests.

6. Cut 5 micron paraffin sections and mount on poly-l-lysine coated slides. Here, too, an additional section for H & E is preferable.

IV. REAGENTS

A. ANTIBODIES

1. Anti-ER IgG (Abbott Labs kit Cat. # 3087-18): use as supplied by the manufacturer. Store in refrigerator at 3°C±3, until the expiration date.

2. Anti-PR IgG (Abbott Labs kit Cat. # 2A09-18): use as supplied by the manufacturer. store in refrigerator at 3°C ±3, until the expiration date.

3. Normal rat IgG(Control in Abbott Laboratory kits Cat. # 3087-18 and # 2A09-18): use as supplied by the manufacturer. Store in refrigerator at 3°C ±3, until the expiration date.

4. Biotinylated goat anti-rat antibody (Zymed Cat. # 62-9540). Store in refrigerator at 3°C ±3, until the expiration date. Use 1:50 dilution (i.e. 20 ul Ab/ml 10% normal goat serum). To this add 50ul/ml of RHEUMATEX latex reagent (Wampole Cat.# 3452) and allow to rotate in the 4°C cold room at least 2 hours or preferably overnight.

5. HRP-Streptavidin (Zymed Cat. # 43-4323). Store in refrigerator at 3°C ±3, until the expiration date. Use 1:500 dilution (i.e.2 ul Ab/ml 10% normal goat serum).

B. OTHER REAGENTS

1. 10% Normal goat serum (NGS): 1ml normal goat serum (Gibco Cat. #200-6210AG) + 9ml phosphate buffered saline (PBS). Store in the refrigerator at 3°C±3 for one week.

2. Phosphate buffered saline (PBS): 800ml double distilled water+ 8.0g NaCl (Sigma Cat.# S-9625)+ 0.2g KCl (Sigma Cat.# P-3911)+ 0.2g KH_2PO_4 (Sigma Cat.# P-5379)+ 2.16g $Na_2HPO_4 \cdot 7H_2O$ (Sigma Cat.# S-9390). Adjust pH between 7.2 & 7.4 and make up to 1000ml. Larger amounts are just multiples of this formula (i.e. 8.0l=64.0g NaCl). Store at room temperature for 6 months. Discard if cloudy or if any precipitate is noted.

3. 0.5% Hydrogen peroxide : 3ml H_2O_2 (Fisher Cat.#H325-500)+ 197ml PBS. Make up fresh day of use and discard after use.

4. TRIS-Magnesium sulfate buffer.pH7.4. The final buffer is 0.05M TRIS-0.01M magnesium sulfate: 661mg Tris-HCL (Sigma Cat.# T-3253) + 97mg Tris base(Sigma Cat# T-1503) + 80ml double distilled water, adjust pH to 7.4. Add 120.4 mg magnesium sulfate(Sigma Cat.# M-7506) and bring up to 100ml. Store at room temperature for six months. Discatd if any bacterial growth is noted.

5. DNase Solution: 5mg DNase (Sigma Cat.# D-5025)/ml TRIS-Mg sulfate. Store DNase below 0°C. Store buffer at room temperature.

6. TRIS-Trypsin buffer pH7.8. The final buffer is 0.05M TRIS: 0.5mg/ml type II porcine trypsin + 1.34mg/ml $CaCl_2$.. Stock solution A: 532mg Tris-HCL + 197mg Tris-base add to 80ml double distilled water and adjust pH to 7.8 Add 134mg $CaCl_2$ and bring up to 100ml double distilled water. Store at room temperature for six months. Discard if any bacterial growth is noted. Stock solution B (10XTrypsin): Dissolve 50mg Type II

porcine Trypsin(Sigma Cat.#T-8128) into 10ml stock solution A. Aliquot 100µl of this solution into 1.5ml Microfuge tubes and store at -20°C. For use add 900µl of stock A to 100µl aliquots of stock B.

7. Rheumatex latex reagent (Wampole Laboratories Cat # 3452). Store in refrigerator at 3°C ±3, until the expiration date.

8. Diaminobenzidine (DAB). (5ml prepared for every 25 slides). Pellets and Substrate supplied with Abbott kits (Cat # 3087-18 and #2A09-18). 1pellet/5ml substrate. Store in refrigerator at 3°C ±3, until the expiration date. DAB IS A SUSPECTED CARCINOGEN USE WITH ADEQUATE VENTILATION AND ALWAYS WEAR GLOVES. (See Standard Operating Procedure for Hazardous Chemicals, LAB SAFETY Section). If the solution is dark brown in color it has deteriorated and must be discarded.

9. Sodium Acetate buffer pH4.0. See step 18B. in procedure. Discard after use.

10. Ethyl Green (CAS Cat.#102700-00) O.5g Ethyl green/100mls sodium acetate buffer. Store at room temperature for 12 months.

11. 100%, 95%, 70% Ethanol. Store sealed at room temperature.

12. Xylene. XYLENE IS TOXIC AND SHOULD BE USED IN A FUME HOOD. (See Standard Operating Procedure for Hazardous Chemicals, LAB SAFETY Section). Store at room temperature.

13. Permount (Fisher Cat # SP 15-100), or other resin based mounting medium. Store at room temperature.

14. Bleach.

C. EQUIPMENT

1. Staining dishes.

2. Humidity chambers (old slide boxes).

3. Microscope slides.

4. PAP pens (provide a wax barrier around the specimen). (The Binding Site Cat.#AD100.5).

5. Cover slips.

6. Eppendorf Pipettemen ([1-5];[5-50];[50-250];[200-1000]).

7. Pipet tips (Phenix T-113).

8. Pasteur pipets.

9. 0.2um Acrodisc.(Gelman No.4192).

10. 3cc Disposable syringe (B-D #9585).

11. Permanent marker pens (American Scientific Product Cat.#P1220).

V. QUALITY CONTROL

A. QUALITY OF SLIDES.

1. Check all slides grossly and microscopically for substandard staining or mounting. This should include inspection for the following:
   a. All numbers within each case are consistent.
   b. All numbers and letters are legible.
   c. No bubbles are retained in the mounting medium.
   d. No "floaters" are present.
   e. No smudged or messy staining.

2. All deficiencies will be corrected as follows:
   a. Cutting, labelling and floaters will be returned to microtomist.
   b. Bubbles and coverslipping errors will be corrected by technician assembling slides.
   c. Poor hematoxylin & eosin staining will be returned to technician running the stain.

B. QUALITY CONTROL OF IMMUNOSTAINING.

1. A positive control should be included with every immunohistochemical staining procedure. This may consist either of a previous specimen known to be positive, or a cell line, such as MCF-7 and T47D, known to be positive. At least 50% of the cells should be positively stained to be deemed acceptable. If the positive control slide is not positive the immunostaining for that day must be repeated.

2. A negative control should be included with every immunohistochemical staining procedure. This should consist of a section stained with normal rabbit IgG as the primary antibody. If it is positive, the staining for that day must be repeated.

3. The quality of all immunostained slides will be assessed by microscopic review of laboratory director. Any inadequately prepared slides will have the procedure repeated immediately.

C. QUALITY CONTROL OF ANTIBODIES:

Each time a new antibody is received, it is checked against the same antibody already utilized, using a known positive control slide.

D. QUALITY IMPROVEMENT.

1. Analysis will be performed with multi-tumor blocks containing breast cancers having known receptor levels to confirm specificity and sensitivity of antibody immunostaining.

2. Unknown samples are received from an outside laboratory (Dr.David Kaminski, Eisenhower Medical Center, Palm Springs, CA) every six months to test our procedures. The results must be in agreement in 100% of the cases. Discrepancies in results are investigated in both laboratories in order to determine the faulty procedure.

VI. PROCEDURE

A. ESTROGEN RECEPTOR

1. Dry the slides in a 60°C oven for at least one hour (to ensure that the sections don't detach from slides). Deparaffinize sections in FRESH xylene (3X5 minutes); rehydrate in graded ethanols (2 minutes each) to PBS.

2. Bleach of endogenous peroxidase activity with 0.5% H_2O_2 in PBS in a staining dish for 15 minutes.

3. Rinse in PBS 2x5 minutes. At this time circle the tissue with PAP pen .

4. Transfer slides to humidity chambers (old slide boxes) and cover with TRIS-Trypsin buffer pH7.8 for 5 minutes at room temperature.

5. Wash slides in running tap water and transfer to PBS.

6. Cover sections with two to three drops of DNase solution and incubate at 37°C for 15 minutues.

7. Rinse inPBS 2X5 minutes.

8. Place slides in humidity chambers (old slide boxes), flood with 10% normal goat serum, using Pasteur pipets, for 20 minutes.

9. Drain excess normal serum from slide. **DO NOT RINSE.** Add primary antibody (2 drops to cover the tissue).

10. Refrigerate overnight.

11. Rinse in PBS (3X5 minutes).

12. Spin down the secondary antibody (3000 rpm , #7, for 5 minutes), then add it to the slides (2 drops to cover the tissue). Incubate for 30 minutes at room temperature.

13. Rinse in PBS (3X5 minutes). At this time make up HRP-streptavidin antibody at 1:500 dilution with 10% normal goat serum.

14. Add HRP-streptavidin (2 drops to cover the tissue). Incubate for 30 minutes at room temperature.

15. Rinse in PBS. 3 X 5minutes. Make up DAB. Draw up DAB in 3 ml disposable syringe and attach 0.22 um millipore filter. **DAB IS A SUSPECTED CARCINOGEN. USE WITH ADEQUATE VENTILATION AND ALWAYS WEAR GLOVES.**

16. Remove slides and blot off excess saline. Add DAB drop-wise through the filter. Incubate 7 minutes at room temperature.

17. Rinse each slide individually with a squirt bottle, letting the DAB drain into a separate dish. Water and bleach should then be added to the dish as well as any left over DAB and the used DAB bottle. These are left to stand until the solution clears and loses color. **This step inactivates the diaminobenzidine.** It then may be safely disposed of.

18. The slides may then be handled in one of two ways:

a. For bright-field viewing without a histologic counterstain:
   1.) Place slides into distilled water.
   2.) Dehydrate through alcohols (70%, 95%, 100%) 1 minute each.
   3.) Xylene 3 x 2 minutes each & mount with Permount.

b. For bright-field viewing with a histologic counterstain:
   1.) Prepare 0.1M Sodium Acetate Buffer pH=4.0 with the following:

Solution (a) 82mls

Solution (b)	18mls
Deionized water	100mls
	200mls

a) 0.2M acetic acid solution (12 mls acetic acid made up to 1000mls with deionized water).
b) 0.2M sodium acetate (27.2g of CH3COONa.3H20 made up to 1000 mls with deionized water).

2.) Prepare an Ethyl Green staining solution by dissolving 0.5 g Ethyl Green in 100mls 0.1 M sodium acetate buffer pH= 4.0

3.) Stain the tissue sections by placing them successively in each of the following for the designated times:

a. 0.1M Sodium acetate buffer pH=4.0	10 minutes.
b. Ethyl Green	10 minutes.
c. Deionized water, 3 times; 10 dips, 10 dips, 30 seconds.	
d. 1-butanol, 3 times; 10 dips, 10 dips, 3 minutes.	
e. Xylene, 3 times;	2 minutes

4.) Coverslip the tissue sections with permount.

B. PROGESTERONE RECEPTOR

1. Dry the slides in a 60°C oven for at least one hour (to ensure that the sections don't detach from the slides). Deparaffinize sections in FRESH xylene (3X5 minutes); rehydrate in graded ethanols (2 min each) and wash in PBS.

2. Bleach of endogenous peroxidase: 0.5% H_2O_2 in PBS for 15 minutes.

3. Wash in PBS (3X5 minutes) at this time circle the tissue with PAP pen.

4. Proceed with slides as in ER step 8 and continue in the same manner.

VII. PROCEDURE NOTES

A. POSSIBLE SOURCES OF ERROR

1. High background staining:
   a. Antibody concentration too high.
   b. Sections allowed to dry out.
   c. Specimen has a lot of endogenous peroxidase (i.e. red blood cells).

Corrective Action:
   a. Lower antibody concentration.
   b. Make sure sections stay moist throughout the procedure.
   c. Ensure correct preparation and usage of 0.5% hydrogen peroxide.

2. No staining:
   a. Antibody inadvertently omitted.
   b. Step inadvertently omitted.
   c. Antibody order inadvertently switched.
   d. DAB substrate outdated.

Corrective Action:
   a. Make sure all antibodies are utilized.
   b. Follow the procedure carefully.
   c. Ensure the use of antibodies in the correct order.
   d. Check expiration date on DAB substrate.

B. HELPFUL HINTS

1. When circling the tissue with the PAP pen go around 2-3 times so that the wax line is very wide. This will help keep an adequate level of saline on the tissue at all times.

2. When washing sections in running water place slides with their backs to the flow of water and make sure that the flow is as low as possible.

3. The humidity chambers should be filled with PBS rather than water so that if a slide happens to fall into it no harm will be done to the tissue.

C. LIMITATIONS

The initial processing of the specimen is critical. Laboratories differ in the way they process paraffin sections and the care they take in handling the specimens. Time of fixation can vary widely from specimen to specimen. Therefore, if a sample is handled poorly or is inadequately fixed, a false negative result can be obtained.

VIII. REFERENCES

1. Greene GL, Nolan C, Engler JP, Jensen EV: Monoclonal antibodies to human estrogen receptor. Proc. Natl. Acad. Sci. USA 77: 5115, 1980.
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PROTO-ONCOGENE HER-2/*neu* IMMUNOHISTOCHEMICAL STAINING PROTOCOL IN PARAFFIN AND FROZEN SECTIONS

I. TIME-LINE OF PROCEDURE USE IN PRESS CONSULTATION LABORATORY.

Adopted: September 25, 1989

Review Policy: Annually since procedure adopted by Dr. Michael Press.

Dates of Review for Last Two Years: January 31,1996 and January 27, 1997, January 26, 1998 and February 5, 1999.

Changes to Procedure Since Adoption: None.

Date of Change to Procedure: None, not applicable.

Most Recent Review of Procedure: January 31, 2000 by Dr. Michael Press.

II. PRINCIPLE

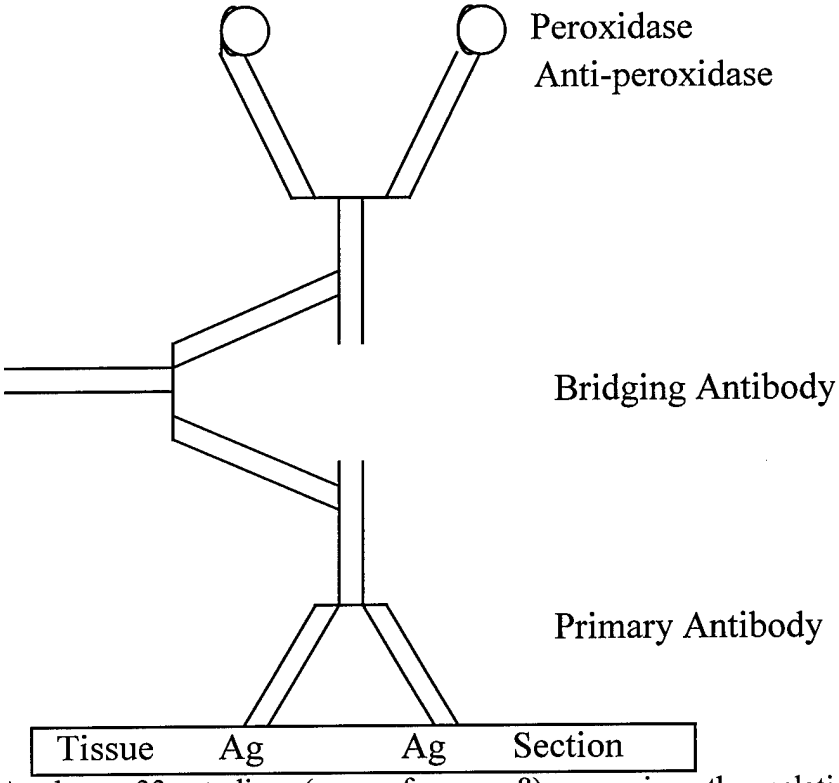
A. REACTION

Rabbit polyclonal antibodies to the protein product of the proto-oncogene HER-2/*neu* (c-*erb* B2) are used with the peroxidase anti-peroxidase immunohistochemical technique to demonstrate this protein product in tissue sections. The immunohistochemical technique involves incubation of the tissue sections with three different antibodies: first is a primary rabbit antibody specific for the antigen, the HER-2/*neu* receptor protein; second is a secondary or bridging antibody directed to rabbit IgG; and third is a rabbit peroxidase antibody with bound peroxidase. The site of immunoprecipitate is identified by the use of a chromogen, diaminobenzidine, that can be visualized microscopically after an enzymatic reaction.

The specificity of the primary antibody for HER-2/*neu* receptor protein has been demonstrated with western immunoblot analyses and competition studies performed using the peroxidase antiperoxidase technique in our laboratory (1).

B. CLINICAL

Amplification of the HER-2/*neu* oncogene is found in approximately 25% of breast cancers (1-4) and is correlated with aggressive biological behavior of the carcinoma as demonstrated by a shorter disease-free interval and shorter overall survival in both axillary lymph node-positive (1-3) and node-negative disease (4,5). The HER-2/*neu* gene codes for a membrane protein that is found at very low levels in normal epithelial cells (6). HER-2/*neu* is closely related to the epidermal growth factor receptor and is, itself, a membrane receptor (7). Gene amplification of HER-2/*neu* is directly correlated with high expression (overexpression) of the gene product at the RNA and protein levels (1,3). Overexpression of the HER-2/*neu* receptor is not only found in all breast cancers showing gene amplification but in approximately 10% of breast cancers lacking gene amplification (3) suggesting another molecular mechanism for increased proein product. Overexpression, like amplification, of HER-2/*neu* is correlated with poor prognosis (1, 3, 8).



At least 33 studies (see reference 8) assessing the relationship of HER-2/*neu* amplification and/or overexpression with relapse and survival in breast cancer have been published. Twenty-six of these studies show a significant association between alteration of the oncogene and outcome for either all patients or subgroups of patients with the disease, while seven claim there is no association. Two of the seven with negative results provide survival curves which show patients with HER-2/*neu* overexpression to have a poorer prognosis although the results failed to achieve statistical significance. Recently, the results from these two studies were combined with a third study of similar size, since the patient material from all three studies had been examined with the same anti-HER-2/*neu* antiserum. The results from this combined reanalysis showed overexpression to be a significant predictor of outcome and led the investigators to conclude that the major problem with the two negative studies was analysis of insufficient numbers of patients. Thus, only five studies in the literature fail to show an association between HER-2/*neu* alteration and breast cancer outcome, while 26 support it.

In addition to studies containing survival data, 21 reports (see reference 8 for citations) have been published on patients for whom there was no or insufficient survival information, but for whom data on other prognostic factors were known. In all twenty-one of these studies, there was a strong association between HER-2/*neu* amplification/overexpression and a variety of other established poor prognostic factors including negative steroid hormone receptors, involved axillary lymph nodes, large tumor size, poor pathologic grade, aggressive histologic subtype, i.e. inflammatory carcinoma, increased DNA ploidy, high mitotic activity and presence of hematogenous metastases. Taken together, the overwhelming majority of published data support the prognostic significance of alterations in this gene in human breast cancer.

The immunohistochemical assay for HER-2/*neu* is very strongly correlated with amplification and overexpression by Northern blot analysis (RNA) and Western blot analysis (protein) (1,3) In addition to the very low rate of false positive or negative results observed with frozen tissue analysis, the immunohistochemical assay has the advantages of speed and small quantity of tissue required for analysis. We have screened 28 different antibodies for sensitivity and specificity in paraffin-embedded specimens (9).

III. SPECIMEN

A. SPECIMEN TYPE

1. There is no patient preparation. Surgical biopsy tissue is frozen as rapidly as practical (within 30 minutes of surgical removal) either by immersion in liquid nitrogen or by freezing in a mechanical freezing unit such as a cryostat. The specimen may be embedded in O.C.T. embedding medium (Miles, Inc.) although this is not required.

2. Paraffin blocks or pre-cut unstained paraffin sections on slides.

B. HANDLING CONDITIONS

1. Refer to SPECIMEN HANDLING in the previous section.

2. The requisition form from the referring hospital laboratory, the frozen tissue container or the paraffin block are labeled with a unique internal laboratory consultation number (corresponding with the laboratory log book), which is used throughout the testing procedures and included in the final report.

3. Sections can be cut in either of two ways:

   a). Cut 4 micron frozen sections and thaw-mount on slides. It is also preferable to cut an additional section for standard H & E staining.

   b) Cut 5 micron paraffin sections and mount on poly-l-lysine coated or superfrost slides. Here, too, an additional section for H & E is preferable.

IV. REAGENTS

A. ANTIBODIES

1. Rabbit anti-Her-2/*neu* (UCLA-Slamon #60 [1]) Store stock solution in refrigerator at 3°C±3 until the expiration date. Diluted solution is made up fresh and discarded after use. Use 1:2000 dilution (i.e. 0.5ul Ab/ml 10% normal goat serum). To this add 50ul/ml of RHEUMATEX latex reagent (Wampole Cat.# 3452) and allow to rotate in the 4°C cold room for at least 2 hours or preferably overnight.

2. Normal rabbit IgG (Pel-Freez Cat# 10101-1) Store stock solution in refrigerator at 3°C±3. Diluted solution is made up fresh and discarded after use. Use 1:1500 dilution (i.e.0.67ul Ab/ml 10% normal goat serum). To this add 50ul/ml of RHEUMATEX latex

reagent (Wampole Cat.# 3452) and allow to rotate in the 4°C cold room for at least 2 hours or preferably overnight.

3. Goat anti-rabbit IgG (Sternberger Monoclonals Incorporated Cat#503). Store stock solution in refrigerator at 3°C±3 until the expiration date. Diluted solution is made up fresh and discarded after use. Use 1:50 dilution (i.e. 20ul Ab/ml 10% normal goat serum). To this add 50ul/ml of RHEUMATEX latex reagent (Wampole Cat.# 3452) and allow to rotate in the 4°C cold room for at least 2 hours or preferably overnight.

4. Rabbit polyclonal PAP (Sternberger Monoclonals Incorporated Cat.#401). Store stock solution in refrigerator at 3°C±3 until the expiration date. Diluted solution is made up fresh and discarded after use. Use 1:50 dilution (i.e. 20ul Ab/ml 10% normal goat serum). To this add 50ul/ml of RHEUMATEX latex reagent (Wampole Cat.# 3452) and allow to rotate in the 4°C cold room at least 2 hours or preferably overnight.

B. OTHER REAGENTS

1. 10% Normal goat serum (NGS): 1ml normal goat serum (Gibco Cat.#200-6210AG) + 9 ml phosphate buffered saline (PBS). Store in the refrigerator at 3°C±3 for one week.

2. Phosphate buffered saline (PBS): 800ml double distilled water+ 8.0g of NaCl (Sigma Cat.# S-9625)+ 0.2g KCl (Sigma Cat.# P-3911)+ 0.2g KH_2PO_4 (Sigma Cat.# P-5379)+ 2.16g $Na_2HPO_4 \cdot 7H_2O$ (Sigma Cat.# S-9390). Adjust pH between 7.2 & 7.4 and make up to 1000ml. Larger amounts are just multiples of this formula (i.e. 8.0L=64.0g NaCl.) Store at room temperature for 6 months. Discard if cloudy or if any precipitate is noted.

3. 1.5% Hydrogen peroxide: 3ml H_2O_2 (Fisher Cat.#H325-500)+ 197 ml PBS. Make up fresh day of use and discard after use.

4. Rheumatex latex reagent (Wampole laboratories Cat.# 3452). Store in refrigerator at 3°C±3, until the expiration date.

5. Diaminobenzidine (DAB). (5ml prepared for every 25 slides). Pellets and Substrate supplied with Abbott kits (Cat.# 3087-18 and # 2A09-18). 1pellet/5ml substrate. Store in refrigerator at 3°C±3, until the expiration date. DAB IS A SUSPECTED CARCINOGEN USE WITH ADEQUATE VENTILATION AND ALWAYS WEAR GLOVES. (See Standard Operating Procedure for Hazardous Chemicals, LAB SAFETY Section). If the solution is dark brown in color it has deteriorated and must be discarded.

6. Sodium Acetate buffer (NaOAc) pH4.0. See step 17 IIa. in PROCEDURE. Discard after use.

7. Ethyl Green (CAS Cat.#102700-00) O.5g Ethyl green/100mls NaOAc buffer. Store at room temperature for 12 months.

8. 100%, 95%, 70% Ethanol. Store sealed at room temperature.

9. Xylene. XYLENE IS TOXIC AND SHOULD BE USED IN A FUME HOOD. (See Standard Operating Procedure for Hazardous Chemicals, LAB SAFETY Section). Store at room temperature indefinitely.

10. Permount (Fisher Cat.# SP 15-100), or other resin based mounting medium. Store at room temperature.

11. Bleach.

12. Water Deionized water from the hall or Sparkletts. See water quality statement (section VIIB7a.)

C. EQUIPMENT

1. Staining dishes.

2. Humidity chambers (old slide boxes).

3. Microscope slides.

4. PAP pens (provide a wax barrier around the specimen). (The Binding Site Cat.#AD100.5)

5. Cover slips.

6. Eppendorf Pipettemen ([1-5];[5-50];[50-250];[200-1000])

7. Pipet tips (Phenix T-113).

8. Pasteur pipets.

9. 0.2um Acrodisc.(Gelman No.4192)

10. 3cc Disposable syringe (B-D #9585).

11. Permanent marker pen (American Scientific Product Cat.#P1220)

V. QUALITY CONTROL

A. QUALITY OF SLIDES.

1. Check all slides grossly and microscopically for substandard staining or mounting. This should include inspection for the following:
   a. All numbers within each case are consistent.
   b. All numbers and letters are legible.
   c. No bubbles are retained in the mounting medium.
   d. No "floaters" are present.
   e. No smudged or messy staining.

2. All deficiencies will be corrected as follows:
   a. Cutting, labelling and floaters will be returned to microtomist.
   b. Bubbles and coverslipping errors will be corrected by technician assembling slides.
   c. Poor hematoxylin & eosin staining will be returned to technician running stainer.

B. QUALITY CONTROL OF IMMUNOSTAINING.

1. A positive control should be included with every immunohistochemical procedure. This may consist either of a previous specimen known to be positive, or a cell line, such

as SKBR3, known to be +++ positive. If the positive control slide is not positive, the entire procedure for the day must be repeated.

2. A negative control should be included with every specimen. This should consist of a section stained with normal rabbit IgG as the primary antibody.

3. The quality of all immunostained slides will be assessed by microscopic review of laboratory director. Any inadequately prepared slides will have the procedure repeated immediately.

C. QUALITY CONTROL OF ANTIBODIES:

Each time a new antibody is received, it is checked against the same antibody already utilized, using a known positive control slide.

D. QUALITY IMPROVEMENT.

Analysis with multi-tumor blocks containing breast cancers with known HER-2/neu gene amplification and expression levels to confirm specificity and sensitivity of antibody immunostaining.

VI. PROCEDURE

1. a) Paraffin slides are dried in a 60°C oven for at least one hour (to ensure that the sections don't come off the slides). Deparaffinize sections in FRESH xylene (3X5 minutes); rehydrate in graded ethanols (2 minutes each) to PBS.

b) For frozen sections start at step 2.

2. Precircle slides with PAP pen.

3. Cut (see Frozen Sectioning) frozen sections 5 um. thick. Thaw mount onto the precircled slides and **place sections immediately in fixative**.

4. Fix slides in 95% EtOH (changed to 100% methanol on 1/1/97) in a staining dish for 15 minutes (optimum time).

5. Hold in PBS buffer in a staining dish until all slides are cut.

6. Bleach off endogenous peroxidase: 0.5%H_2O_2 in PBS in a stainining dish for 15 minutes.

7. Rinse in PBS 2 X 5 minutes.

8. Transfer slides to humidity chambers (old slide boxes), flood with 10% normal goat serum (or normal serum from whatever the host animal of the bridging antibody was) using Pasteur pipets, for 20minutes. [Paraffin sections may be circled with PAP pen now]. At this time spin down the primary antibody in the table-top centrifuge at 3000rpm (#7) for 5 minutes.

9. Drain excess normal serum from slide. DO NOT RINSE. Add primary antibodies (2 drops to cover the tissue). Incubate one hour at room temperature. [Paraffin sections are incubated overnight in refrigerator]. Record day's run in log book.

10. Rinse in PBS 3 X 5 minutes. Spin down the bridging antibody at 3000 rpm (#7) for 5 minutes.

11. Remove slides from the last rinse and blot off the excess saline. Fastidious wiping is not necessary nor preferable at this time as the saline will recede from the circled area by itself and too much blotting tends to dry out the tissue. THE TISSUE SHOULD REMAIN MOIST AT ALL TIMES. Add bridging antibody to the sections (2 drops to cover the tissue). Incubate for 30 minutes at room temperature.

12. Rinse in PBS 3 X 5minutes. Spin down the Peroxidase Anti-Peroxidase antibody at 3000 rpm (#7) for 5 minutes.

13. Remove slides and blot off excess saline. The slides may be re-grouped according to block at this time. This avoids confusion when you're mounting. Add the Peroxidase Anti-Peroxidase antibody (2 drops to cover the tissue). Incubate for 30 minutes at room temperature.

14. Rinse in PBS 3 X 5minutes. Make up DAB. CAUTION; DAB IS A SUSPECTED CARCINOGEN HANDLE APPROPRIATELY. 1 tablet/5ml substrate from Abbott kit. Draw up DAB in 3ml disposable syringe and attach 0.22um millipore filter.

15. Remove slides and blot off excess saline. Add DAB drop-wise through the filter. Incubate for 7 minutes at room temperature.

16. Rinse each slide individually with a squirt bottle, letting the DAB drain into a separate dish. Water and bleach should then be added to the dish as well as any left over DAB and the bottle it was in. This can be left to stand until the solution clears and loses color. **This step inactivates the diaminobenzidine.** It then may be safely disposed of.

17. The slides may then be handled in one of two ways:

I. For bright-field viewing without a histologic counterstain:
a) Place slides into distilled water and dehydrate through alcohols 1 minute each.
b) Xylene 2 minutes each & mount with Permount.

II. For bright-field viewing with a histologic counterstain:
a)Prepare 0.1M Sodium Acetate Buffer pH=4.0
i-0.2M acetic acid (12 mls acetic acid made up to 1000mls with deionized water.)
ii-0.2M sodium acetate (27.2g of CH3COONa.3H20 made up to 1000 mls with deionized water.

Solution (i)	82mls
Solution (ii)	18mls
deionized water	100mls
	200mls

Ethyl Green solution: dissolve 0.5 g Ethyl Green in 100mls
0.1 M sodium acetate buffer pH= 4.0

b) Staining procedure:
i-0.1M Sodium acetate buffer pH=4.0 10 minutes.
ii-Ethyl Green 10 minutes.
iii-Deionized water, 3 times; 10 dips, 10 dips, 30 seconds.
iv-1-butanol, 3 times; 10 dips, 10 dips, 3 minutes.
v- Xylene, 3 times; 2 minutes each.
vi-Mount.

VII. PROCEDURE NOTES

A. POSSIBLE SOURCES OF ERROR

1. High background staining:
   a. Antibody concentration too high.
   b. Sections allowed to dry out.
   c. Specimen has a lot of endogenous peroxidase (i.e. red blood cells).

Corrective action:
   a. Lower antibody concentration.
   b. Make sure sections stay moist throughout the procedure.
   c. Ensure correct preparation and usage of 0.5% hydrogen peroxide.

2. No staining:
   a. Antibody inadvertently omitted.
   b. Step inadvertently omitted.
   c. Antibody order inadvertently switched.
   d. DAB substrate outdated.

Corrective action:
   a. Make sure all antibodies are utilized.
   b. Follow the procedure carefully.
   c. Ensure the use of antibodies in the correct order.
   d. Check expiration date on DAB substrate.

B. HELPFUL HINTS

1. When circling the tissue with the PAP pen go around 2-3 times so that the wax line is very wide. This will help keep an adequate level of saline on the tissue at all times.

2. When washing sections in running water place slides with their backs to the flow of water and make sure that the flow is as low as possible.

3. The humidity chambers should be filled with PBS rather than water so that if a slide happens to fall into it no harm will be done to the tissue.

C. LIMITATIONS

1. In general, frozen tissue stains much more readily than paraffin-embedded tissue, however the morphology of frozen sections is much more problematic. Breast tissue with its high fat content is usually extremely difficult to cut. Therefore a separate (preferably serial) section should be cut with all frozen tissues and stained with H(hematoxylin) and E(eosin).

2. The initial processing of the paraffin-embedded specimen is critical. Laboratories differ in the way they process paraffin sections and the care they take in handling the

specimens. Time of fixation can vary widely from specimen to specimen. Therefore, if a sample is handled poorly or is inadequately fixed, a false negative result can be obtained.

VIII. METHOD PERFORMANCE SPECIFICATIONS.

The performance specifications of the HER-2/*neu* immunohistochemical staining method in 187 paraffin-embedded breast cancers was determined first by characterizing the sensitivity and specificity of 28 different HER-2/*neu* antibodies relative to gene amplification and overexpression determined by Southern hybridization, Northern hybridization, Western immunoblot analysis and immunohistochemistry. The Figure below summarizes the findings showing which antibodies were most sensitive (toward the left) and which antibodies were the least sensitive (toward the right). The findings were published in Cancer Research, volume , pages 2771-2777, 1994. (See following pages for a reprint.) The antibody that we use is one of the two most sensitive antibodies.

Figure. Bar graph comparing the percentage of breast cancers immunostained in each of four HER-2/*neu* expression categories by 28 different antibodies. One of the antibodies, TAB250, was used with (TAB250p) and without (TAB250) protease pretreatment of tissue sections. Two forms of another antibody, A8010, were used either as fresh antiserum (A8010) or as lyophilized antiserum that was reconstituted (A8010[l]). Note the variation in the positive percentage of stained cases with different antibodies.

The four expression categories were based on HER-2/*neu* gene amplification and expression results obtained by analysis of DNA by Southern hybridization, RNA by northern hybridization, total protein by western immunoblot and frozen tissue sections by immunohistochemistry. These same 187 breast cancers were analyzed (graph above) as paraffin-embedded tissues. The figure legend identifies three groups of breast cancers with overexpression, separately categorized by gene amplification levels (>5-fold, 2 to 5-fold, and 1H), and a group of breast cancers with neither amplification nor overexpression of HER-2/*neu* (1Low). Immunostained cases are presented as a percentage of the breast cancers identified in each breast cancer expression group (ordinate).

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Secondly, we wished to confirm that our HER-2/*neu* immunostaining results were correlated with an increased risk of recurrent disease in women with axillary lymph node-

negative breast cancer. We used the #60 antibody to analyze HER-2/*neu* expression in 105 breast cancer cases with recurrent disease and in 105 matched control breast cancers with no recurrent disease during a 5-year clinical follow-up period. The results of this study published in Cancer Research (volume 53: 4960-4970, 1993) confirmed that HER-2/*neu* overexpression by immunohistochemical staining was associated with an 3-fold increased risk of recurrent disease (P-value = 0.001). HER-2/*neu* overexpression was independent of other known prognostic markers. **From this data it was estimated that at 10 years follow-up approximately 22% of women with primary node-negative breast cancer having low expression of HER-2/*neu* (no overexpression) will experience recurrent disease; whereas approximately 34% of women whose primary breast cancer has moderate expression (overexpression) will experience recurrent disease; and approximately 73% of women whose primary breast cancer has moderate expression (overexpression) will experience recurrent disease** (*Cancer Res.* 53: 4960-4970, 1993).

IX. REFERENCES

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P53 Tumor Suppressor
Immunohistochemical Staining Protocol
for Paraffinized Tissue

I. TIME-LINE OF PROCEDURE USE IN PRESS CONSULTATION LABORATORY.

Adopted: June 28, 1994

Review Policy: Annually since procedure adopted by Dr. Michael Press.

Dates of Review for Last Two Years: January 30,1996, January 27, 1997, January 26, 1998 and February 5, 1999.

Changes to Procedure Since Adoption: None.

Date of Change to Procedure: None, not applicable.

Most Recent Review of Procedure: January 30, 2000 by Dr. Michael Press.

II. PRINCIPLE

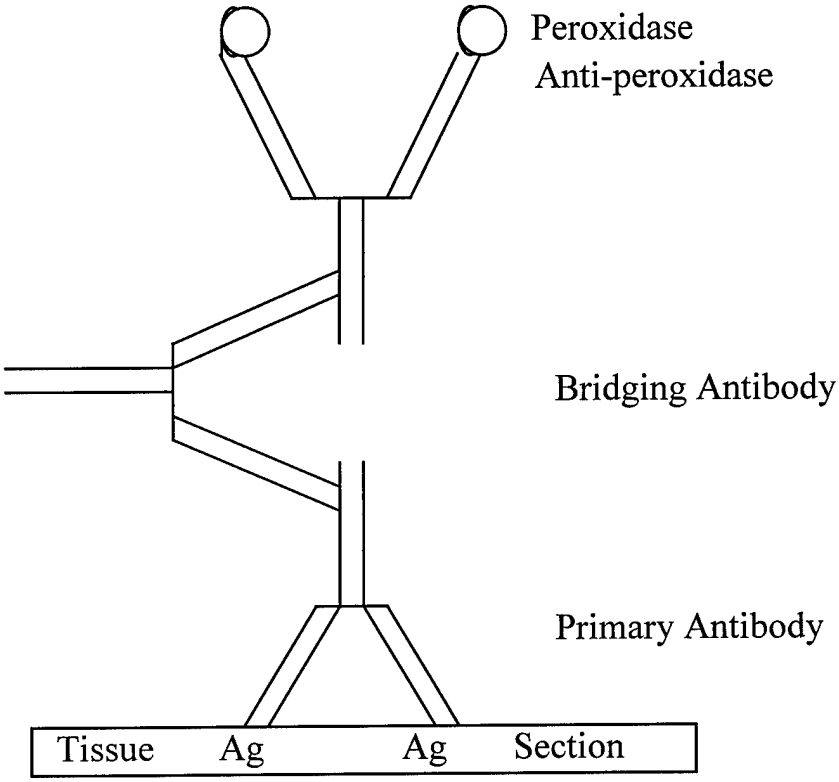
A. REACTION

Mouse monoclonal antibodies to the protein product of the tumor suppressor gene p53 are used with the peroxidase anti-peroxidase immunohistochemical technique to demonstrate this protein product in tissue sections. The immunohistochemical technique involves incubation of the tissue sections with three different antibodies: first is a primary antibody specific for the p53 protein product, second is a secondary or bridging antibody directed to the primary antibody, and third is an antiperoxidase antibody with bound peroxidase (PAP). The site of immunoprecipitate is identified by the use of a chromogen, diaminobenzidine (DAB), that can be visualized microscopically after an enzymatic reaction.

The specificity of the primary antibody for p53 protein product has been demonstrated with western immunoblot analyses .

B. CLINICAL

P53 is to date the most commonly mutated gene in human cancers, and is one of the star members of the tumor suppressor gene family. Of the 6.5 million people diagnosed with cancer each year worldwide, about half have p53 mutations in their tumors. Among common tumors, about 70% of colorectal cancers, 50% of lung cancers, and 40% of breast cancers carry p53 mutations. Several methods to screen for p53 mutations have been used, and they include SSCP, HOT, CDGE, PCR and Direct Sequencing, RNase protection, and immunohistochemistry. Normal p53 protein product has a short half-life, thus the insufficient accumulation of the p53 product does not allow detection by immunostaining. However, the mutated p53 mRNA has a longer half-life and the protein product therefore accumulates in the cell nucleus at a highter concentration so it can be detected selectively over wildtype cells by immunostaining. Detection of p53 mutations in cells has clinical importance because it allows early diagnosis, as in the case of Li-Fraumeni Syndrome, and can serve as a prognostic marker for cancer. For example, in the case of patients whose breast cancer has not invaded nearby lymph nodes, studies have shown that only about 25% of these women will relapse. P53 may be used as a prognostic marker to identify women with a less-favorable clinical outcome. In primary breast cancer p53 immunostaining has been correlated with both a shorter disease-free interval and a shorter overall survival (1-6).



III. SPECIMEN

A. SPECIMEN TYPE

There is no patient preparation. Paraffin blocks or pre-cut unstained paraffin sections on slides.

B. HANDLING CONDITIONS

1. Refer to SPECIMEN HANDLING in the previous section.

2. The requisition form from the referring hospital laboratory and the paraffin block are labeled with a unique internal laboratory consultation number (corresponding to that in the laboratory logbook), which is used throughout the testing procedures and included in the final report.

3. Cut (see Paraffin Sectioning) 4-micron paraffin sections and mount on poly-l-lysine coated or superfrost slides. An additional section for H & E is preferable.

IV. REAGENTS

A. ANTIBODIES

1. Primary antibody: Monoclonal Mouse Anti-Human p53 Protein (DAKO-p53, DO-7; Code No. M7001). Store stock solution in refrigerator at 3°C±3 until the expiration date. Use 1:100 dilution in normal rabbit serum. To this add 50ul/ml of Rheumatex latex reagent and allow to rotate in the 4°C cold room for at least 2 hours or preferably overnight.

2. Primary antibody for negative control: normal mouse IgG (Zymed Laboratories Inc. Cat.#02-6502). Store stock solution in refrigerator at 3°C±3 until the expiration date. Diluted solution is made up fresh and discarded after use. Use normal mouse IgG at 1:1000 dilution (i.e. 1ul Ab/ ml 10% normal rabbit serum). To this add 50ul/ml of Rheumatex latex reagent and allow to rotate in the 4°C cold room for at least 2 hours or preferably overnight.

3. Secondary antibody: Rabbit anti-mouse IgG (Zymed Laboratories Inc. Cat.#61-6500). Store stock solution in refrigerator at 3°C±3 until the expiration date. Diluted solution is made up fresh and discarded after use. Use 1:50 dilution (i.e. 20ul Ab/ml 10% normal rabbit serum). To this add 50ul/ml of Rheumatex latex reagent and allow to rotate in the 4°C cold room for at least 2 hours or preferably overnight.

4. Tertiary antibody: Mouse ClonoPAP (Sternberger Monoclonals Incorporated Cat.#405). Store stock solution in refrigerator at 3°C±3 until the expiration date. Diluted solution is made up fresh and discarded after use. Use 1:50 dilution (i.e. 20ul Ab/ml 10% normal rabbit serum). To this add 50ul/ml of Rheumatex latex reagent and allow to rotate in the 4°C cold room at least 2 hours or preferably overnight.

B. OTHER REAGENTS

1. 10% Normal rabbit serum (NRS): 1ml rabbit serum (Gibco BRL Cat.#16120-016) + 9ml phosphate buffered saline (PBS). Store in the refrigerator at 3°C±3 for one week. Discard if turbid.

2. Phosphate buffered saline (PBS): 800ml double distilled water+ 8.0g of NaCl (Sigma Cat.#S-9625)+ 0.2g KCl (Sigma Cat.#P-3911)+ 0.2g KH_2PO_4 (Sigma Cat.#P-5379)+ 2.16g $Na_2HPO_4 \cdot 7H_2O$ (Sigma Cat.#S-9390). Adjust pH between 7.2 & 7.4 and make up to 1000ml. Larger amounts are just multiples of this formula (i.e. for 8.0L need 64.0g NaCl.). Store at room temperature for 6 months. Discard if cloudy or if any precipitate is noted.

3. 1.5% Hydrogen peroxide: 3ml of 30%H_2O_2 (Fisher Cat.#H325-500)+ 197ml PBS if using 19 slides or less. Make up fresh the day of use and discard after use.

4. Rheumatex latex reagent (Wampole Laboratories Cat.# 34S2). Store in refrigerator at 3°C±3 until the expiration date.

5. Diaminobenzidine (DAB). Pellets and substrate reagent supplied with Abbott kits (Cat.#3087-18 and #2A09-18). 1pellet/5ml substrate reagent. Store in refrigerator at 3°C±3 until the expiration date. DAB IS A SUSPECTED CARCINOGEN; USE WITH ADEQUATE VENTILATION AND ALWAYS WEAR GLOVES. (See Standard Operating Procedure for Hazardous Chemicals, LAB SAFETY Section). If the solution is dark brown in color it has deteriorated and must be discarded.

6. Sodium acetate buffer (NaOAc) pH4.0. See step 17 IIa. in PROCEDURE. Discard after use.

7. Ethyl Green (CAS Cat.#102700-00) O.5g Ethyl Green/100mls NaOAc buffer. Store at room temperature for 12 months.

8. 100%, 95%, 70% Ethanol. Store sealed at room temperature.

9. Xylene. XYLENE IS TOXIC AND SHOULD BE USED IN A FUME HOOD. (See Standard Operating Procedure for Hazardous Chemicals, LAB SAFETY Section). Store at room temperature indefinitely.

10. Permount (Fisher Cat.# SP 15-100), or other resin based mounting medium. Store at room temperature.

11. Bleach.

12. For antigen retrieval: 10mM Citrate buffer (pH 6.0): 2.1g Citric acid Monohydrate (Sigma Cat #. 1909) ad 1L. adjust pH to 6.0 with approximately 13 ml 2M NaOH.

13. Water See water quality statement (section VIIB7a.)

C. EQUIPMENT

1. Staining dishes.

2. Humidity chambers (old slide boxes).

3. Microscope slides.

4. PAP pens (to provide a wax barrier around the specimen). (The Binding Site Cat.#AD100.5)

5. Cover slips.

6. Eppendorf Pipettemen ([1-5];[5-50];[50-250];[200-1000])

7. Pipet tips (Phenix T-113).

8. Pasteur pipets.

9. 0.22um millipore filter (Millex-GV Cat.#SLGVR25KS) or 0.2um Acrodisc.millipore filter (Gelman No.4192)

10. 3cc Disposable syringe (B-D #9585).

11. Permanent marker pen (American Scientific Product Cat.#P1220)

12. For antigen retrieval:
    a. Microwave oven.
    b. Plastic Coplin jars.

V. QUALITY CONTROL

A. QUALITY OF SLIDES.

1. Check all slides grossly and microscopically for substandard staining or mounting. This should include inspection for the following:
    a. All numbers within each case are consistent.
    b. All numbers and letters are legible.
    c. No bubbles are retained in the mounting medium.
    d. No "floaters" are present.
    e. No smudged or messy staining.

2. All deficiencies will be corrected as follows:
    a. Cutting, labelling and floaters will be returned to microtomist.
    b. Bubbles and coverslipping errors will be corrected by technician assembling slides.
    c. Poor hematoxylin & eosin staining will be returned to technician running stainer.

B. QUALITY CONTROL OF IMMUNOSTAINING.

1. A positive control should be included with every immunohistochemical procedure. This may consist either of a previous specimen known to be +++ positive, or a cell line, such as SKBR3, known to be positive. If the positive control slide is not positive, the entire procedure for the day must be repeated(see Procedure Notes).

2. A negative control should be included with every specimen. This should consist of a section stained with normal rabbit IgG or normal mouse IgG as the primary antibody.

3. The quality of all immunostained slides will be assessed by microscopic review of laboratory director. Any inadequately prepared slides will have the procedure repeated immediately.

C. QUALITY CONTROL OF ANTIBODIES:

Each time a new antibody is received, it is checked against the same antibody already utilized, using a known positive control slide.

D. QUALITY IMPROVEMENT.

Analysis with multi-tumor blocks containing breast cancers with known p53 gene mutation to confirm specificity and sensitivity of antibody immunostaining.

VI. PROCEDURE

1. Dry slides in a 60°C oven for at least one hour (to ensure that the sections don't come off the slides). Deparaffinize sections in FRESH xylene (three times; 5 minutes each). Rehydrate in graded ethanols (twice in 100% EtOH, twice in 95% EtOH, and twice in 70% EtOH; 2 minutes each) to PBS.

2. Bleach off endogenous peroxidase: 0.5% H_2O_2 in PBS in a staining dish for 15 minutes.

3. Rinse in PBS 2 X 5 minutes.

4. Transfer slides to humidity chambers (old slide boxes). Circle tissue sections with a PAP pen. Flood tissue sections with 10% normal rabbit serum using Pasteur pipets. Incubate for 20minutes. At this time spin down the primary antibody in the table-top centrifuge at 3000rpm (#7) for 5 minutes.

5. Drain excess normal serum from the slides. DO NOT RINSE. Add primary antibody (2 drops to cover the tissue).

6. Refrigerate overnight.

7. Rinse in PBS 3 X 5 minutes. Spin down the bridging antibody at 3000rpm (#7) for 5 minutes.

8. Remove slides from the last rinse and blot off the excess saline. Fastidious wiping is not necessary nor preferable at this time as the saline will recede from the circled area by itself and too much blotting tends to dry out the tissue. THE TISSUE SHOULD REMAIN MOIST AT ALL TIMES. Add bridging antibody to the sections (2 drops to cover the tissue). Incubate for 30 minutes at room temperature.

9. Rinse in PBS 3 X 5minutes. Spin down the Peroxidase Anti-Peroxidase antibody at 3000rpm (#7) for 5 minutes.

10. Remove slides and blot off excess saline. The slides may be re-grouped according to block at this time. This avoids confusion when you're mounting. Add the Peroxidase Anti-Peroxidase antibody (2 drops to cover the tissue). Incubate for 30 minutes at room temperature.

11. Rinse in PBS 3 X 5minutes. Prepare DAB (5ml for every 25 slides). CAUTION: DAB IS A SUSPECTED CARCINOGEN HANDLE APPROPRIATELY. Draw up DAB in 3ml disposable syringe and attach 0.22um millipore filter.

12. Remove slides and blot off excess saline. Add DAB drop-wise through the filter. Incubate for 7 minutes at room temperature.

13. Place bleach in a new dish. Put leftover DAB and the flask containing it into the bleach. After 7 minutes, drain DAB from the sections into bleach. DAB will turn brown upon contact with bleach. This can be left to stand until the solution clears and loses color. **This step inactivates the diaminobenzidine.** It then may be safely disposed of.

14. The slides may then be handled in one of two ways:

I. For bright-field viewing without a histologic counterstain:
a) Place slides into distilled water and dehydrate through graded alcohols 1 minute each.
b) Place in xylene three times 2 minutes each & mount with Permount.

II. For bright-field viewing with a histologic counterstain:
a) Prepare 0.1M sodium acetate buffer pH=4.0
First make
i-0.2M acetic acid (12ml acetic acid made up to 1000mls with deionized water)
ii-0.2M sodium acetate (27.2g of $NaCH_3COO \cdot 3H_20$ made up to 1000ml with deionized water)

Add Solution (i)	82ml
Solution (ii)	18ml
deionized water	100ml
	200ml

b) Prepare Ethyl Green solution:
Dissolve 0.5g Ethyl Green in 100ml 0.1M sodium acetate buffer
c) Staining procedure:

i-0.1M sodium acetate buffer	10 minutes.
ii-Ethyl Green	10 minutes.
iii-Deionized water, 3 times;	10 dips, 10 dips, 30 seconds.
iv-1-butanol, 3 times;	10 dips, 10 dips, 3 minutes.
v-Xylene, 3 times;	2 minutes each.
vi-Mount.	

VII. PROCEDURE NOTES

A. POSSIBLE SOURCES OF ERROR

1. High background staining:
a. Antibody concentration too high.
b. Sections allowed to dry out.
c. Specimen has a lot of endogenous peroxidase (i.e. red blood cells).

Corrective action:
a. Lower antibody concentration.

b. Make sure sections stay moist throughout the procedure.
c. Ensure correct preparation and usage of 1.5% hydrogen peroxide.

2. No staining:
   a. Antibody inadvertently omitted.
   b. Step inadvertently omitted.
   c. Antibody order inadvertently switched.
   d. DAB substrate outdated.

Corrective action:

a. Make sure all antibodies are utilized.
b. Follow the procedure carefully.
c. Ensure the use of antibodies in the correct order.
d. Check expiration date on DAB substrate.

B. HELPFUL HINTS

1. When circling the tissue with the PAP pen go around 2-3 times so that the wax line is very wide. This will help keep an adequate level of saline on the tissue at all times.

2. When washing sections in running water place slides with their backs to the flow of water and make sure that the flow is as low as possible.

3. The humidity chambers should be filled with PBS rather than water so that if a slide happens to fall into it no harm will be done to the tissue.

C. LIMITATIONS

1. In general, frozen tissue stains much more readily than paraffin-embedded tissue; however, the morphology of frozen sections is much more problematic. Breast tissue with its high fat content is usually extremely difficult to cut. Therefore a separate (preferably serial) section should be cut with all frozen tissues and stained with H(hematoxylin) and E(eosin).

2. The initial processing of the paraffin-embedded specimen is critical. Laboratories differ in the way they process paraffin sections and the care they take in handling the specimens. Time of fixation can vary widely from specimen to specimen. Therefore, if a sample is handled poorly or is inadequately fixed, a false negative result can be obtained.

VIII. METHOD PERFORMANCE SPECIFICATIONS.

Confirmation of the immunohistochemical staining method was conducted in three phases. First, breast cancers were characterized for p53 mutations. Second, 6 different antibodies were tested for their ability to immunostain P53 in breast cancers. Third, the mutations and immunostaining results were unblinded and compared with one another.

1.) P53 Mutation Analysis. Two different groups of breast cancers were analyzed for p53 mutations; one was a series of 55 breast cancers and the other was a group of 98 bilateral breast cancer specimens. The series of 55 breast cancers, available as both frozen specimens and paraffin-embedded specimens, were analyzed for p53 mutations by DNA sequencing of the entire open reading frame using cDNA derived from tumor mRNA. Eleven of the 55 cases (20%) had mutations and 44 were wild-type (80%).

Ninety-eight breast tumors from 49 women were analyzed for p53 mutations. Forty-one pairs were synchronous while eight pairs were asynchronous (interval between diagnoses less than one month or 48 months or longer). Genomic DNA from these breast cancers was

analyzed for possible mutations in exons 5 - 8. The reverse transcriptase - polymerase chain reaction (PCR) with single-strand conformational polymorphism technique was used to screen for mutations and DNA sequencing was used to confirm and to characterize the mutations. Thirteen tumors (13%) from 11 women contained mutations in exons 5 - 8 of the p53 gene. One tumor had two mutations; thus a total of 14 mutations were identified. All mutations were single-base changes or point mutations, three of which did not change the amino acid sequence of the protein. Seven mutations were found in exon 5, 3 in exon 7, and 4 in exon 8.

2.) P53 Immunostaining. The series of 55 breast cancers were immunostained in a blinded fashion with each of seven different antibodies (DO-7, DO-1, 1801, CM1, CM10, BP53, and 421) both with and without antigen retrieval. The best immunostaining (most intense with highest percentage of positive tumor cells) was obtained with antigen retrieval; however, the immunostaining with antigen retrieval did not correlate with p53 mutations and had many false-positive results. Twenty-two (40%) of the 55 breast cancers had positive nuclear immunostaining for p53. None of the cases had cytoplasmic immunostaining. The percentage of positively immunostained tumor cells in these 22 cases varied from 12% to 98%.

The second breast cancer series, from women with bilateral breast cancer, were immunostained with DO-7 antibody, the antibody which gave the best correlation in the previous series of 55 cases (see below).

3.) Comparison of P53 Immunostaining and p53 Mutations. For the series of 55 breast cancers with sequencing of the entire p53 open reading frame, D0-7 antibody without antigen retrieval yielded the strongest correlation with mutant p53. In order to confirm that this result could be duplicated, a second set of breast cancers from women with bilateral breast cancer was analyzed. Our preliminary results indicated that false negative immunostained cases represent a significant proportion (greater than 10%) of mutant cases. Interestingly, some cases with negative immunostaining had mutations of exons 5 - 8. One case had a splice junction mutation at codon 292. Another case had two mutations one, a missense mutation, coded for the substitution of serine for cysteine at codon 215 and the other, a small deletion of three base pairs, resulted in an in-frame deletion of codon 217. One mutation outside of exons 5 to 8 was a deletion of a single base pair in codon 147 which resulted in a frame-shift mutation.

The series of 98 bilateral breast cancers analyzed for mutations included 62 breast cancers for which both DNA sequencing results and immunostaining were available. Among these, 40 tumors (65%) had neither nuclear immunostaining nor mutations and 7 (11%) had both mutations and positive staining for p53. There were 4 tumors which had a DNA mutation but lacked immunostaining and 11 which were immunostained but lacked p53 mutations. **There was a statistically significant correlation between the presence of p53 mutations and positive immunostaining for p53 protein (Fischer Exact Test, two sided, $p < 0.01$).** For additional details see the manuscript "Clonal analysis of bilateral breast cancer" (10).

Why p53 overexpression is observed in some breast cancers which apparently lack p53 mutations is unresolved at this time. These false positive immunostaining cases, lacking any alteration in the open reading frame, represent an interesting group. The prevailing explanation for positive immunostaining is that mutations increase the stability of mutated p53 protein and increase its half-life causing accumulation in the nucleus (106). An alternative hypothesis could account for positive immunostaining with either mutated or wild-type p53. p53 expression could be down-regulated by a negative feed-back loop provided by one or more of the gene products transcriptionally activated by p53. When this feed-back is disrupted, either by alteration of p53 or by alteration of a p53-activated protein, then the negative feedback is disrupted and p53 expression is not down-regulated. Therefore, p53 would be expressed at high levels detectable with immunohistochemistry. If this were correct, then cases having wild-type p53 and positive immunostaining would be expected to have an alteration in other proteins whose expression is induced by p53, such as WAF1/Cip1 (p21), GADD45, MDM2, FLN retroviral element and muscle creatine kinase.

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HER-2/*neu* PROTO-ONCOGENE
FLUORESCENCE *IN SITU* HYBRIDIZATION (FISH) PROTOCOL (Oncor, Inc.)
IN TISSUE SECTIONS

I. TIME-LINE OF PROCEDURE USE IN LABORATORY FOR BREAST CANCER ANALYSIS.

Adopted: April 20, 1998

Review Policy: Annually since procedure adopted by Dr. Michael Press.

Dates of Review for Last Two Years: January 12,1999.

Changes to Procedure Since Adoption: Changed from the Oncor FISH assay to the Vysis FISH assay (see protocol description which follows).

Date of Change to Procedure: Discontinued the Oncor FISH assay on December 18, 1998.

Most Recent Review of Procedure: January 26, 2000 by Dr. Michael Press.

II. PRINCIPLE.

A. REACTION

Fluorescence *in situ* hybridization (FISH) of the HER-2/*neu* gene involves hybridization of a specific DNA probe with sequences complementary to the gene to permit assessment of the number of copies of this gene in each cell nucleus. FISH technology combines the advantages of direct gene amplification assessment with direct localization in morphologically identified tumor cells. FISH is applicable to tumors of all sizes because studies can be performed on sections from the original specimen blocks used for diagnosis. In many samples, direct comparison can be made with FISH assays on normal cells from the same preparation. Further, if amplification were localized rather than diffusely distributed within a tumor, it would be detectable by FISH but could be diluted below detectable limits in extracted tumor DNA required for other procedures.

The Oncorl INFORM" HER-2/*neu* Gene Detection System is a kit consisting of DNA probe and detection reagents that yields a green fluorescent signal at the site of the HER-2/*neu* gene, on a blue fluorescent background of stained nuclear DNA. The kit is intended to be used with sections (4 μm) of formalin-fixed, paraffin-embedded human breast cancer tissue.

Briefly, the methodology is as follows. Sections of formalin-fixed, paraffin-embedded breast cancer tissue mounted on microscope slides are pretreated chemically (Pretreatment Step, reduction of peptide disulfide bonds) and enzymatically (Protein Digestion Step, digestion of proteins) to remove proteins that block DNA access. The DNA in the sections is converted from double- to single-strand by solution denaturation at 75°C using a mixture of 20X SSC (saline sodium citrate) and formamide. A hybridization solution, containing labeled DNA probe which is complementary to the HER-2/*neu* gene sequence, is applied to the tissue section, which is then incubated under conditions favorable for annealing of probe DNA and genomic DNA sequences. Unannealed probe is washed off using a mixture of 20X SSC and formamide. The hybridized probe is detected using a fluorescently-tagged ligand (fluorescein-labeled avidin) which binds to the label on the DNA probe, thereby immobilizing the fluorescein at the site of the HER-2/*neu* gene. The remainder of the DNA is then stained with an intercalating fluorescent counterstain (DAPI in Antifade). Excitement of fluorescein and DAPI by light from a mercury arc lamp with appropriate filters in an epifluorescence microscope results in the emission of green and blue light, respectively. The observer selects for these two colors by using a microscope filter set designed for simultaneous viewing of DAPI and fluorescein, and scores nuclei in the tissue section for the number of green signals on a blue background.

B. CLINICAL

Among all cancers in the U.S.. breast cancer is expected to be the most common cancer (32% of all cancers / 182,000 newly diagnosed cases per year) in women and to be the second most common cause of death from cancer (18% / 46,000 deaths per year) (4). After surgery, breast cancers with positive axillary nodes, which account for 30% of all breast cancers (5), are associated with a shorter disease-free survival (6, 7) and a shorter overall survival (8) than node negative breast cancers. It has been generally accepted that patients with breast cancer and positive axillary nodes at diagnosis, should be offered adjuvant systemic treatment.

The HER-2/*neu* proto-oncogene has been shown by us (9-13) and by others (14-18) to be amplified and/or overexpressed in 20 - 30% of breast cancers, ovarian cancers, endometrial cancers, gastric carcinomas and salivary gland carcinomas. Human tumors show a strong correlation between HER-2/*neu* gene amplification and overexpression (11, 13, 19). HER-2/*neu* gene amplification and/or overexpression is correlated with shorter disease-free interval and shorter overall survival in axillary lymph node-negative breast cancers (9, 12), axillary lymph node-positive breast cancers (19), ovarian carcinomas (19), endometrial carcinomas (13) and salivary gland carcinomas (11). HER-2/*neu* gene amplification and/or overexpression has also been correlated with responsiveness to various forms of adjuvant therapy including high-dose adriamycin chemotherapy (20), taxol chemotherapy (Press, unpublished data) and tamoxifen anti-hormone treatment (21). In addition, clinical trials have shown that approximately 10% to 20% of women with metastatic breast cancer having HER-2/*neu* overexpression respond to treatment with Herceptin, a humanized monoclonal anti-p185$^{\text{HER-2/}neu}$ antibody recently approved by the U.S. Food and Drug Administration.

III. SPECIMEN

A. SPECIMEN TYPE

1. Paraffin blocks or pre-cut unstained paraffin sections on slides.

B. HANDLING CONDITIONS

1. Refer to SPECIMEN HANDLING in the previous section.

2. The requisition form from the referring hospital laboratory, the paraffin block are labeled with a unique internal laboratory consultation number (corresponding with the laboratory log book), which is used throughout the testing procedures and included in the final report.

3. Sections are cut as follows:

   a) Cut 4 micron paraffin sections and mount on poly-l-lysine coated or superfrost slides. The first section is for hematoxylin & eosin staining.

IV. REAGENTS

A. MATERIALS PROVIDED.

The Oncor INFORM HER-2/*neu* assay kit contains the following components sufficient for 20 assays:

Oncor INFORM HER-2/*neu* S8000-Kit Component List

COMPONENT	PART No.	VOLUME	STORAGE	STABILITY
Pretreatment Powder	S8000-1	60 g (3 x 20 g)	2°C to 25°C	18 Months as powder; Use on same day as reconstitution
Protein Digesting Enzyme	S8000-2	50 mg (2 x 25 mg)	-15°C to -25'C	18 Months Total Unopened and Reconstituted

2OX SSC	S8000-3	220 ml	2°C to 25°C	18 Months
Formamide	S8000A	320 ml	2°C to 8°C	18 Months
10X PBD	S8000-5	200 ml	2°C to 8°C	18 Months
Biotinylated HER-2/neu DNA Probe	58000-6	200 µl	- 1 5°C to -2 5°C	18 Months
Blocking Reagent One	S8000-8	2.4 ml	2'C to 8'C 18 Months	
Detection Reagent				
(Fluorescein-labeled Avidin) 18 Months	S8000-9	2.4 ml	2°C to 8°C	
Blocking Reagent Two 18 Months	S8000-1 0	1.2 ml	2°C to 8°C	
Anti-Avidin Antibody 18 Months	58000-11	1.2 ml	2°C to 8°C	
DAPI/Antifade	S8000-12	1.0 ml	- 1 5°C to -25°C	18 Months
Antifade	S8000-13	200 pl	-1 5°C to -25°C	18 Months
Plastic Coverslips	S8000-14	96 coverslips	2°C to 25°C	18 Months

B. OTHER REAGENTS

1. Distilled, deionized water
2. A minimum of 4 ml sterile deionized water
3. Ethanol: 70%, 80%, 90% and 100%
4. Xylene
5. 6N HCI

C. LABORATORY SUPPLIES.

1. Silanized microscope slides (OncorCat. No. S1308) or equivalent
2. Glass coplin jars - plastic coplin jars (50 ml) may be used during all steps not performed in a waterbath
3. Glass coverslips (25 x 25 mm and 24 x 50 mm)
4. Micropipettor tips
5. Microcentrifuge tubes
6. Microcentrif uge tube rack and float
7. Serological pipets, 5 ml, 10 ml and 25 ml

8. Pipette bulbs for serological pipettes
9. 50 ml Polypropylene tubes
10. Graduated cylinders; 100 ml, 500 ml and 1000 ml
11. Liter storage bottles (4)
12. Latex, vinyl or nitrile gloves
13. Shaved or crushed ice
14. Forceps
15. Timer
16. Laboratory tissues or paper towels
17. Other lab supplies as needed

D. EQUIPMENT NEEDED.

1. Humidified Chambers
2. Incubator at 37°C ±2°C
3. Oven at 65°C ±5°C
4. Waterbaths capable of maintaining temperatures from 37°C to 78°C at a tolerance of ±2°C.
5. Ice Bath
6. Calibrated thermometers
7. Adjustable Micropipettors: 0-20 µl, 0-100 µl or 0-200 µl and 0-1000 µl
8. Vortex
9. pH meter
10. Balance capable of accurately weighing 12.0 g
11. Microcentrifuge

An epifluorescence microscope equipped with specific filters is required for viewing the processed specimens.

NOTE: The microscope must be located in a darkened room and equipped property for optimal visualization of the fluorescein signal. Light leakage from the lamp housing of the microscope should be minimized.

1. 100W mercury arc light source
2. DAPI/FITC/Texas Red triple band pass filter set (see table below)
3. DAPI filter set (see table below)
4. FITC/Texas Red dual band pass filter set (see table below)
5. lOx dry, 40x dry or oil, and 100x oil fluorescence objectives
6. Non-fluorescing immersion oil

V. REAGENT PREPARATION.

1. Pretreatment Solution (30%)
   Prepare 40 ml of Pretreatment Solution (30%) per coplin jar used by:

12.0 g Pretreatment Powder (S8000-1) (must be weighed out)
30 ml Deionizedwaterdissolveat43'Cfor5-10minutes
Adjust to 40 ml Deionizedwater to 4O ml
Prepare fresh for each assay on the day of use.

2. 2X SSC

Prepare 2X SSC by adding:
180 ml 2OX SSC (S8000-3)
1620 ml Deionized water
1800 ml Total

Adjust the pH of the 2X SSC to 7.0 with 6N HCI. This reagent may be prepared in advance and stored in a glass or plastic vessel at 18°C to 25°C until the expiration date of the 2OX SSC Reagent. Check and adjust the pH to 7.0 before use, if necessary.

3. Protein Digesting Enzyme Stock Solution.

Protein Digesting Enzyme is provided in two (2) bottles, each containing 25 mg of enzyme, To properly prepare Protein Digesting Enzyme Stock Solution, the entire contents of each bottle must be rehydrated. Prepare by adding:

1 ml Sterile Deionized water
25 mg (1 bottle) Protein Digesting Enzyme (S8000-2)
1 mL Total

Rehydrate both bottles of Protein Digesting Enzyme at the same time. Add the Sterile Water directly to each enzyme bottle and shake thoroughly until dissolved. Aliquot the rehydrated Protein Digesting Enzyme into 5 aliquots of 400 µl each, dispensed into microcentrifuge tubes. This stock solution may be prepared in advance and stored at -15°C to -25°C until the expiration date of the unreconstituted Protein Digesting Enzyme.

4. Protein Digesting Enzyme Working Solution.

Prepare Protein Digesting Enzyme Working Solution by adding:

400 µl Protein Digesting Enzyme Stock Solution
40 ml 2X SSC
40.4 ml Total

Add 40 ml of 2X SSC to a 50 ml capped disposable centrifuge tube, and prewarm to 37°C in a water bath. Thaw an aliquot of Protein Digesting Enzyme Stock Solution

in the 37°C water bath and add to the prewarmed 2X SSC. Mix thoroughly before use. Prepare fresh each time, immediately before use.

5. Denaturation Solution

Prepare 40 ml of Denaturation Solution (70% Formamide/2X SSC, pH 7.0) per coplin jar, by adding:

4 ml	2OX SSC (S8000-3)
8 ml	Deionized water
28 ml	Formamide (S8000-4)
40 ml	Total

Prepare fresh for each assay on the day of use, adjusting the pH to 7.0 with 6N HCl.

6. Post-Hybridization Wash Solution

Prepare 40 ml of Post-Hybridization Wash Solution (50% Formamide/ 2X SSC, pH 7.0) per coplin jar, by adding:

4 ml	2OX SSC (S8000-3)
16 ml	Deionized water
20 ml	Formamide (S8000-4)
40 ml	Tota I

Prepare fresh for each assay on the day of use, adjusting the pH to 7.0 with 6N HCl.

7. 1X PBD

10X PBD settles into two phases during shipping. In order to prepare 1X PBD properly, the 10X PBD must be mixed well prior to dilution. To prepare measure:

200 ml	10X PBD (S8000-5)
add 1800 ml	Deionizedwater
2000 ml	Total

This reagent may be prepared in advance and stored in a glass or plastic vessel at 2°C to 8°C until the expiration date of the 10X PBD Reagent.

VI. STEP-BY-STEP PROCEDURE

A. ASSAY SET-UP

DAY 1:

1. Adjust one water bath to 37°C±2°C, another to 43°C±2°C, and a third to 75°C to 78°C (see NOTE following step 4.1 in Section 4, " DENATURATION").
2. Prepare 40 ml of Pretreatment Solution (30%) and pre-warm to 43°C.
3. Pre-warm 40 ml of 2X SSC to 37°C±2°C in a glass coplin jar.
4. Prepare 40 ml of Denaturation Solution and pre-warm to 75°C to 78°C (see NOTE following step 4.1 in Section 4, "DENATURATION").
5. Prepare two (2) graded ethanol series, 40 ml each of 70%, 80%, 90%, and 100% Ethanol. Pre-chill one series to -20°C±5°C, and store at -20°C±5°C until use; maintain the other series at room temperature, or, transfer a single series to -20°C after post digestion ethanol series.
6. Pre-warm Incubator and Humidified Chamber to 37°C±2°C.

DAY 2:

1. Adjust water baths to 37°C ±2°C and 43°C ±2°C.
2. Pre-warm 2 coplin jars with 40 ml of 2X SSC to 37°C±2°C.
3. Prepare 40 ml of PoSt-Hybridization Wash Solution and pre-warm to 43°C ±2°C.
4. Pre-warm 400 ml of 1 X PBD to 18°C to 25°C.
5. Pre-warm Humidified Chamber to 37°C ± 2°C.

DAY 1

B. PRETREATMENT

1. Pretreat slides by immersing a maximum of 4 slides in a coplin jar containing 40 ml of pre-warmed 30% Pretreatment Solution in a 430C 2'C water bath for 15 minutes.
2. Wash slides in 40 ml of 2X SSC at room temperature for 1 minute. Repeat wash twice using fresh 2X SSC.
3. Dehydrate slides through the room-temperature graded series of ethanois for 2 minutes in each of 70%, 80%, 90% and 1 00% ethanol.
4. Allow slides to air dry inclined with label end down.

C. PROTEIN DIGESTING ENZYME TREATMENT

1. Prepare 40 ml of Protein Digesting Enzyme Working Solution as directed above, using 40 ml of 37°C±2°C prewarmed 2X SSC. Prepare this Working Solution immediately before use.
2. Immerse a maximum of 4 slides in a coplin jar of prewarmed Protein Digesting Enzyme Working Solution and incubate at 37°C±2°C for 40 minutes.
3. Wash slides in 40 ml of 2X SSC at room temperature for 1 minute. Repeat wash twice using fresh 2X SSC.
4. Dehydrate slides through the room-temperature graded series of ethanols for 2 minutes in each grade of ethanol: 70%, 80%, 90% and 100%.
5. Allow slides to air dry inclined with label end down.

NOTE: If the tissue is underdigested after hybridization and microscopic review, it can be exposed to further proteinase digestion. Refer to Step 10, Extended Protein Digestion.

D. DENATURATION

NOTE: Time and temperature are of critical importance for preserving tissue morphology and attachment to the slide. Verify the temperature of the Denaturation Solution with a clean calibrated thermometer inserted directly into the coplin jar.

1. Denature slides by immersing in a coplin jar containing 40 ml of prewarmed Denaturation Solution in the 75°C water bath for 8 minutes.

NOTE: If multiple slides are processed simultaneously, addition of each slide will cause the solution to drop 1°C. Therefore, the temperature of the solution must be adjusted up 1°C from 75°C for each additional slide to be simultaneously added to denaturation solution. Denature no more than 4 slides per coplin jar at a time.

2. Immediately transfer slides to the pre-chilled (-20°C ±5°C) 70% ethanol and rinse for 2 minutes. Repeat rinse in pre-chiiled (-20°C ±5°C) 80%, 90%, and 100% ethanol solutions, successively.

3. Allow slides to air dry inclined with label end down.

E. HYBRIDIZATION

1. Probe Preparation:
   1.1. Prewarm the HER-21neu DNA probe at 37°C ±2°C for 5 minutes.
   1.2. Vortex for 1 minute and centrifuge 2 to 3 seconds to collect contents in the bottom of the tube.
   NOTE: Do not heat denature this probe by boiling or immersion in the 75'C water bath.
2. Pipette l0 µl of probe solution onto the denatured tissue section and cover gently with a 25 mm x 25 mm glass coverslip. Larger tissue sections may require up to 20 µl of probe and larger glass coverslips.

   NOTE: 10 µl of probe will adequately cover a section of up to 25 mm x 25 mm area. Larger tissue sections will require proportionately more probe, reducing the number of assays performed by each kit.

   NOTE: Do not seal the coverslips with rubber cement, as this may lead to tissue damage when removing the coverslip after hybridization.

3. Incubate at 37°C±2°C for 12 to 16 hours in a humidified chamber.

DAY 2

F. POST-HYBRIDIZATION WASHING

1. Carefully remove the coverslip by sliding it to the side and lifting the overhanging edge with forceps.
2. Wash slides in a coplin jar containing 40 ml of pre-warmed PostHybridization Wash Solution in the 43°C ±2°C water bath for 15 minutes.
3. Wash slides in a coplin jar containing 40 ml of pre-warmed 2X SSC in the 37°C water bath with frequent agitation for 10 minutes. Repeat the wash with fresh 2X SSC.
4. Place slides in a coplin jar containing 40 ml of 1X PBD at 18°C to 25°C.

NOTE: Do not allow slides to dry beyond this step. If necessary, slides can be stored at 2°C to 8°C in 40 ml of 1X PBD for up to 2 weeks before detection.

G . DETECTION

NOTE: Remove Blocking Reagent One, Detection Reagent, Blocking Reagent Two, and Anti-Avidin Antibody from 2°C to 8°C storage and place on ice. Just prior to use, mix by gently inverting each bottle several times to evenly resuspend the components.

1. Remove slides from 1X PBD and briefly blot excess fluid from the edge.

NOTE: Do not allow the slide sample surface to become dry. This will cause high background fluorescence.

2. Apply 60 µl of Blocking Reagent One to each slide and place a plastic coverslip over the solution. incubate 5 minutes in a humidified chamber at room temperature.

3. Peel off the plastic coverslip and blot the coverslip dry for re-use. Tilt the slide and allow fluid to drain briefly.

4. Apply 60 µl of Detection Reagent to each slide and replace the plastic coverslip over the solution. Incubate 20 minutes in a humidified chamber at room temperature. After 10 minutes of the 20 minute incubation, lift and replace the plastic coverslip to ensure even fluid distribution.

5. Peel off the plastic coverslip and discard. Wash slides in a coplin jar containing 40 ml of 1X PBD at room temperature for 2 minutes. Repeat this wash 2 times using fresh 1X PBD.

H. SIGNAL AMPLIFICATION

1. Remove slides from 1X PBD, tilt the slide and allow fluid to drain briefly, then briefly blot excess fluid from the edge.
2. Apply 60 µl of Blocking Reagent Two to each slide and place a fresh plastic coverslip over the solution. Incubate 5 minutes in a humidified chamber at room temperature.
3. Peel off the plastic coverslip and blot the coverslip dry for reuse. Tilt the slide and allow fluid to drain briefly.
4. Apply 60 µl of Anti-Avidin Antibody to each slide and replace the plastic coverslip over the solution. Incubate 20 minutes in a humidified chamber at room temperature. At 10 minutes of incubation, lift and replace the plastic coverslip to ensure even fluid distribution.
5. Peel off the plastic coverslip and discard. Wash slides in a coplin jar containing 40 ml of 1 X PBD at room temperature for 2 minutes. Repeat thic Wash 2 times using fresh IX PBD.
6. Apply 60 pl of Blocking Reagent One to each slide and place a fresh plastic coverslip over the solution. Incubate 5 minutes in a humidified chamber at room temperature.
7. Peel off the plastic coverslip and blot the coverslip dry for reuse. Tilt the slide and allow fluid to drain briefly.
8. Apply 60 µl of Detection Reagent to each slide and replace the plastic coverslip over the solution. Incubate 20 minutes in a humidified chamber at room temperature. After 10 minutes of incubation, lift and replace the plastic coverslip to ensure even fluid distribution.
9. Peel off the plastic coverslip and discard. Wash slides in a coplin jar containing 40 ml of 1X PBD at room temperature for 2 minutes. Repeat this wash 2 times using fresh 1X PBD.

I. NUCLEAR COUNTERSTAINING

1. Counterstain nuclei by adding 20 µl of DAPI/Antifade to each slide. Cover with 24 x 50 mm glass coverslip.

   NOTE: After applying Counterstain, the coverslip may tend to slip. To prevent this, blot edges of slide on paper towels to remove excess fluid.

2. Proceed to the Quality Control and then the Interpretation sections for analysis. Stained slides may be stored in the dark at -15°C to -25'C for up to five days before analysis. However, for best results with the brightest signal, the slides should be viewed immediately after staining. The slides may be

viewed for approximately one hour with the microscope before the signal fades.

J. EXTENDED PROTEIN DIGESTION

OPTIONAL STEP: Proceed with Extended Protein Digestion if under-digestion occurs and is determined to interfere with interpretation of assay results.

NOTE 1: If there are more prepared specimen slides, the procedure can be repeated using a longer protein digestion time at Step 3.2. If the degree of under-digestion is judged to be minimal (ie., there is persistent autofluorescence but it does not appear to be very thick), an extra 20 minutes of digestion, for a total of 60 minutes would be appropriate.

NOTE 2: Having autofluorescence could necessitate doubling the protein digestion time to 80 minutes. If the sample is limited, it is possible to repeat the procedure on the same slide starting with the Protein Digestion Enzyme Working Solution, Step 3.

1. Remove the coverslip by gently wiping off the immersion oil with tissue paper and soaking the slide in 40 ml 2X SSC, pH 7.0 in a coplin jar at room temperature until the coverslip falls off.
2. Transfer the slide to a coplin jar containing fresh 2X SSC, pH 7.0 for several minutes to clean off any residual DAPI/Antifade.
3. Soak slides in prewarmed Protein Digestion Enzyme Working Solution at 37°C$\pm$ 2°C. The effect of this protein digestion and the initial digestion is cumulative. Twenty (20) additional minutes of digestion might be an appropriate starting time for tissue that seems very underdigested after the initial 40 minute digestion.
4. Wash slides in 40 ml 2X SSC, pH 7.0 room temperature with agitation for 1 0 seconds.
5. Dehydrate slides in 70%, 80%, 90%, and 100% ethanol at room temperature for 1 minute each. Allow slides to air dry.
6. Continue by repeating steps 4.1 to end of the remainder of the procedure.

VII. QUALITY CONTROL

A. QUALITY OF SLIDES.

1. Check all slides grossly and microscopically for substandard staining or mounting. This should include inspection for the following:
   a. All numbers within each case are consistent.
   b. All numbers and letters are legible.
   c. No bubbles are retained in the mounting medium.
   d. No "floaters" are present.
   e. No smudged or messy staining.

2. All deficiencies will be corrected as follows:
   a. Cutting, labelling and floaters will be returned to microtomist.
   b. Bubbles and coverslipping errors will be corrected by technician assembling slides.
   c. Poor hematoxylin & eosin staining will be returned to technician running stainer.

B. QUALITY CONTROL OF HYBRIDIZATION.

1. Internal control benign and normal cells should be inspected to confirm that the nuclei of these cells contain the normal number of HER-2/neu signals (2) and the normal number of chromosome 17 centromeres.

2. An amplification control, for example SK-BR-3 human breast cancer cells, should be included with procedure.

3. The quality of all immunostained slides will be assessed by microscopic review of laboratory director. Any inadequately prepared slides will require that the procedure be repeated.

4. If control slides fail to meet the slide acceptance criteria, the assay may not have been performed properly or the PathVysion Kit component(s) may have performed inadequately. A repeat analysis with fresh control slides and patient specimen slide(s) will be necessary.

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HER-2/*neu* PROTO-ONCOGENE
FLUORESCENCE *IN SITU* HYBRIDIZATION (FISH) PROTOCOL (Vysis, Inc.)
IN TISSUE SECTIONS

I. TIME-LINE OF PROCEDURE USE IN LABORATORY FOR BREAST CANCER ANALYSIS.

Adopted: December 19, 1998.

Review Policy: Annually since procedure adopted by Dr. Michael Press.

Dates of Review for Last Two Years: January 12,1999.

Changes to Procedure Since Adoption: Changed from the Oncor FISH assay to the Vysis FISH assay on December 19, 1998 (see protocol description which follows).

Date of Change to Procedure: Discontinued the Oncor FISH assay on December 18, 1998.

Most Recent Review of Procedure: January 26, 2000 by Dr. Michael Press.

II. PRINCIPLE.

A. REACTION

The PathVysion HER-2 DNA Probe Kit (Vysis, Inc.) is designed to detect the HER-2/*neu* gene via fluorescence *in situ* hybridization (FISH) in formalin-fixed, paraffin-embedded human breast cancer tissue specimens. The fluorescence *in situ* hybridization (FISH) technique has been used to detect HER-2/*neu* gene amplification in human breast carcinoma cell lines in both interphase and metaphase cells (1-3). FISH appears to be an alternative technique capable of overcoming many of the inherent technical and interpretative limitations of other techniques. For quantification of HER-2/*neu* gene amplification, FISH assesses not only the level of HER-2/*neu* gene amplification directly in the tumor cells while retaining the characteristic morphology of the tissue studied, but also the spatial distribution of oncogene copies in individual uncultured primary breast carcinomas.

The LSI HER-2/*neu* DNA probe is a 190 Kb SpectrumOrange directly labeled fluorescent DNA probe specific for the HER-2/*neu* gene locus (17ql 1.2-ql2). The CEP 17 DNA probe is a 5.4 Kb SpectrumGreen directly labeled fluorescent DNA probe specific for the alpha satellite DNA sequence at the centromeric region of chromosome 17 (17p11.1-q11.1). The probes are pre-mixed and pre-denatured in hybridization buffer for ease of use. The assay is rapid, non-radioactive, requires little tumor material, and is capable of detecting as few as 2 to 8 copies of the oncogene.

B. CLINICAL BACKGROUND INFORMATION.

Among all cancers in the U.S.. breast cancer is expected to be the most common cancer (32% of all cancers / 182,000 newly diagnosed cases per year) in women and to be the second most common cause of death from cancer (18% / 46,000 deaths per year) (4). After surgery, breast cancers with positive axillary nodes, which account for 30% of all breast cancers (5), are associated with a shorter disease-free survival (6, 7) and a shorter overall survival (8) than node negative breast cancers. It has been generally accepted that patients with breast cancer and positive axillary nodes at diagnosis, should be offered adjuvant systemic treatment.

The HER-2/*neu* proto-oncogene has been shown by us (9-13) and by others (14-18) to be amplified and/or overexpressed in 20 - 30% of breast cancers, ovarian cancers, endometrial cancers, gastric

carcinomas and salivary gland carcinomas. Human tumors show a strong correlation between HER-2/*neu* gene amplification and overexpression (11, 13, 19). HER-2/*neu* gene amplification and/or overexpression is correlated with shorter disease-free interval and shorter overall survival in axillary lymph node-negative breast cancers (9, 12), axillary lymph node-positive breast cancers (19), ovarian carcinomas (19), endometrial carcinomas (13) and salivary gland carcinomas (11). HER-2/*neu* gene amplification and/or overexpression has also been correlated with responsiveness to various forms of adjuvant therapy including high-dose adriamycin chemotherapy (20), taxol chemotherapy (Press, unpublished data) and tamoxifen anti-hormone treatment (21). In addition, clinical trials have shown that approximately 10% to 20% of women with metastatic breast cancer showing HER-2/*neu* overexpression respond to treatment with Herceptin, a humanized monoclonal anti-p185$^{HER\text{-}2/neu}$ antibody recently approved by the U.S. Food and Drug Administration.

III. SPECIMEN

A. SPECIMEN TYPE

1. Paraffin blocks or pre-cut unstained paraffin sections on slides.

B. HANDLING CONDITIONS

1. Refer to SPECIMEN HANDLING below.
2. The requisition form from the referring hospital laboratory and the paraffin block are labeled with a unique internal laboratory consultation number (corresponding with the laboratory log book), which is used throughout the testing procedures and included in the final report.
3. Sections are cut as follows:

   a) Cut 5 micron paraffin sections and mount on poly-l-lysine coated or superfrost slides. The first section is for hematoxylin & eosin staining.

IV. REAGENTS

A. MATERIALS PROVIDED.

The Vysis PathVision kit contains sufficient reagents to process approximately 20 assays. An assay is defined as one 22 mm x 22 mm target area.

1) LSI HER-2/*neu* SpecTrumOrange (low copy number *E. coli* vector) / CEP 17 SpectrumGreen DNA Probe *(E. coli* plasmid)
   Vysis P.N.: 30-171060
   Quantity: 200 uL
   Storage: -20°C in the dark
   Composition: SpectrumGreen fluorophore-labeted alpha satellite DNA probe for chromosome 17, SpectrumOrange fluorophore-labeled DNA probe for the HER-2/*neu* gene locus, and blocking DNA, pre-denatured in hybridization buffer.

2) DAPI Counterstain
   Vysis P.N.: 30-804840
   Quantity: 300 µL

Storage: -20°C in the dark
Composition: 1000 ng/mL DAPI (4,6-diamidino-2-phenylindole) in phenylenediamine dihydrochloride, glycerol, and buffer.

3) NP-40
Vysis P.N.: 30-804820
Quantity: 4 mL (2 vials)
Storage: -20 to 25°C
Composition: NP-40

4) 2OX SSC salts
Vysis P.N.: 30-805850
Quantity: 66 g for up to 250 mL of 20X SSC solution
Storage: -20° to 25°C
Composition: sodium chloride and sodium citrate

Storage and Handling
Store the unopened PathVysion Kit as a unit at -20°C protected from light and humidity. The 20X SSC salts and NP-40 may be stored separately at room temperature. Expiration dates for each of the unopened components are indicated on the individual component labels. These storage conditions apply to both opened and unopened components.

B. OTHER REAGENTS, REQUIRED BUT NOT PROVIDED.

1. Paraffin Pretreatment Reagent Kit (Vysis Cat. # 32-801200), which includes:
   Pretreatment Solution (NASCN) Quantity: 5 x 50 mL
   Protease (Pepsin, 2500-3000 units/mg) Quantity: 5 x 25 mg
   Protease Buffer (NaCl solution, pH 2) Quantity: 5 x 50 mL
   Wash Buffer (2X SSC, pH 7) Quantity: 2 x 250 mL
2. Neutral buffered formalin solution (Sigma product no. F4136)
3. Hemo-De clearing agent (Fisher Product No. 15-182-507A)
4. Hematoxalin and eosin (H & E)
5. Immersion oil appropriate fluorescence microscopy. Store at room temperature.
6. Ultra-pure, formamide. Store at 4°C for up to one month from delivery (See manufacturer's recommendations for detailed information).
7. Ethanol (100%). Store at room temperature.
8. Concentrated (12N) HCI
9. 1N NaOH
10. Purified water (distilled or deionized or Milli-Q). Store at room temperature.
11. Rubber cement
12. Drierite and Nitrogen gas

C. EQUIPMENT

Precleaned silanized or positively charged glass microscope slides
Slide warmer (45 - 50°C)
22 mm x 22 mm glass coverslips
Microliter pipettor (1-10 µl) and sterile tips
Polypropylene microcentrifuge tubes (0.5 or 1.5 mL)
Timer
Microtome
Magnetic stirrer
Vortex mixer
Microcentrifuge
Graduated cylinder

Water baths (37±1°C, 72±1°C, and 80±1°C)
Protein-free water bath (40°C)
Air incubators (37°C and 56°C)
Diamond-tipped scribe
Humidified hybridization chamber
Forceps
Disposable syringe (5 mL)
Coplin jars (6) Suggested type: Wheaton Product No. 900620 vertical staining jar
Fluorescent microscope equipped with recommended filters (see next section)
pH meter and pH paper
Calibrated thermometer
Microscope slide box with lid
0.45 pm pore filtration unit

Microscope Euipment and Accessories

Microscope: An epi-illumination fluorescence microscope is required for viewing the hybridization results.

Excitation Light Source: A 100 watt mercury lamp with life maximum of about 200 hours is the recommended excitation source. Record the number of hours that the bulb has been used and replace the bulb before it exceeds the rated time. Ensure that the lamp is properly aligned.

Obiectives: Use oil immersion fluorescence objectives with numeric apertures ≥ 0.75 when using a microscope with a 100 watt mercury lamp. A 40X objective, in conjunction with 10X eyepieces is suitable for scanning. For FISH analysis, satisfactory results can be obtained with a 63X or 100X oil immersion achromat type objective.

Immersion Oil: The immersion oil used with oil immersion objectives should be one formulated for low auto fluorescence and specifically for use in fluorescence microscopy.

Filters: Multi-bandpass fluorescence microscope filter sets optimized for use with the CEP and LSI DNA probe kits are available from Vysis for most microscope models. The recommended filter sets for the PathVysion Kit are the DAPI/9-Orange dual bandpass, DAPI/Green dual bandpass and DAPI/Green/Orange triple bandpass. Hybridization of the LSI HER-2/*neu* and CEP 17 probes to their target regions is marked by orange and green fluorescence, respectively. All of the other DNA will fluoresce blue with the DAPI stain.

V. PROCEDURE

A. PREPARATION OF WORKING REAGENTS

1. 20X SSC (3M sodium chloride, 0.3M sodium citrate, 12H 5.3)

To prepare 20X SSC pH 5.3, add together:

66 g	20X SSC
200 mL	purified water
250 mL	final volume

Mix thoroughly. Measure pH at room temperature with a pH meter. Adjust pH to 5.3 with concentrated HCl. Bring the total volume to 250 mL with purified water. Filter through a 0.45 µm pore filtration unit. Store at room temperature for up to 6 months.

2. Denaturing Solution (70% formamide / 2X SSC, pH 7.0-8.0)

To prepare denaturing solution. add together:

49 mL	formamide
7 mL	2OX SSC, pH 5.3

14 mL	purified water
70 mL	final volume

Mix thoroughly, measure pH at room temperature using a pH meter with glass pH electrode to verify that the pH is between 7.0 - 8.0. This solution can be used for up to one week. Check pH prior to each use. Store at 2 - 8°C in a tightly capped container when not in use.

3. Ethanol Solutions

Prepare v/v dilutions of 70%, 85%, and 100% using 100% ethanol and purified water. Dilutions may be used for one week unless evaporation occurs or the solution becomes diluted due to excessive use. Store at room temperature in tightly capped containers when not in use.

4. Post-Hybridization Wash Buffer (2X SSC/0.3% NP 40)

To prepare, add together:

100 mL	2OX SSC, pH 5.3
847 mL	Purified water
3 mL	NP-40
1000 mL	Final Volume

Mix thoroughly. Measure pH at room temperature using a pH meter. Adjust pH to 7.0 - 7.5 with 1N NaOH. Adjust volume to 1 liter with purified water. Filter through 0.45 µm pore filtration unit. Discard used solution at the end of each day. Store unused solution at room temperature for up to 6 months.

B. SPECIMEN PROCESSING AND SLIDE PREPARATION.

1. Specimen Collection and Processing

The PathVysion Kit is designed for use on formalin-fixed, paraffin-embedded tissue specimens. Tissue collections should be performed according to the laboratory's standard procedures. Selection of tissue for PathVysion assay should be performed by the M.D. laboratory associate in consultation with the laboratory director. Exposure of the specimens to acids, strong bases, or extreme heat, should be avoided. Such conditions are known to damage DNA and may result in FISH assay failure.

Breast tissue should be prepared in sections between 4 and 6 microns thick. Formalin-fixed, paraffin-embedded tissue may be handled and stored according the laboratory's routine procedures. To ensure optimum results from the PathVysion Kit, these methods should be consistent for all specimens analyzed. To identify target areas, H & E staining should be conducted on every 10th slide of the same tissue block.

Tissue sections should be mounted on the positive side of an organosilane-coated slide in order to minimize detachment of the tissue from the slide during FISH assay. The PathVysion Kit contains reagents sufficient for approximately 20 assays; one assay for the PathVysion Kit is defined as a 22 mm x 22 mm area. Larger specimen sections will require more than 10 µl of probe per assay.

2. Slide Preparation from Formalin-Fixed, Paraffin-Embedded Tissue

The following method may be used for preparing slides from formalin-rixed, paraffin-embedded tissue specimens:

1. Cut 4-6 µm thick paraffin sections using a microtome.
2. Float the sections in a protein-free water bath at 40°C.

3 . Mount the section on the positive side of an organosilane-coated slide.
4. Allow slides to air dry.
5. Bake slides overnight at 56°C.

3. Slide Pretreatment

Slides must be deparaffinized and the specimens fixed prior to assay with the PathVysion Kit. Tne package insert for the Vysis Paraffin Pretreatment Reagent Kit (Product No. 32-801200) contains detailed instructions. The following is a brief description of the procedure.

a. Deparaffinizing Slides.

- Immerse slides in Hemo-De for 10 minutes at room Temperature.
- Repeat twice using new Hemo-De each time.
- Dehydrate slides in 100% ETOH for 5 minutes at room temperature. Repeat.
- Air dry slides or place slides on a 45-50°C slide warmer.

b. Pretreating slides.

- Immerse slides in 0.2N HCl for 20 minutes.
- Immerse slides in purified water for 3 minutes.
- Immerse slides in Wash Buffer for 3 niinuees.
- Immerse slides in Pretreatment Solution at 80°C for 30 minutes.
- Immerse slides in purified water for 1 minute.
- Immerse slides in Wash Buffer for 5 minutes. Repeat.

c. Protease Treatment.

- Remove excess buffer by blotting edges of the slides on a paper towel.
- Immerse slides in Protease Solution at 37°C for 10 minutes.
- Immerse slides in Wash Buffer for 5 minutes. Repeat.
- Dry slides on a 45-50°C slide warmer for 2-5 minutes.

d. Fixing the Specimen.

- Immerse the slides in neutral buffered formalin at room temperature for 10 minutes.
- Immerse the slides in wash buffer for 5 minutes. Repeat.
- Dry slides on a 45-50'C slide warmer for 2-5 minutes.
- Proceed with the PatbVysion assay protocol.

C. ASSAY PROCEDURE.

1. Fluorescence *In Situ* Hybridization Procedure Summary

a. Denaturation of Specimen DNA

The timing for preparing the probe solutions should be carefully coordinated with denaturing the specimen DNA so that both will be ready for the hybridization step at the same time.

- Prewarm the humidified hybridization chamber (an air-tight container with a piece of damp blotting paper or paper towel approximately 1 in. x 3 in. taped to the side of the container) to 37'C by plucing it in the 37'C incubator prior to slide preparation. Moisten

the blotting paper or paper towel with water before each use of the hybridization chamber.

- Verify that the pH of the denaturing solution is 7.0 - 8.O at room temperature before use. Add denaturing solution to Coplin jar and place in a 72 +1 °C water bath for at least 30 minutes. or until the solution temperature reaches 72±1°C. Verify the solution temperature before use.
- Mark the areas to be hybridized with a diamond-tipped scribe.
- Denature the specimen DNA by immersing the prepared slides in the denaturing solution at 72±1 °C (≤6 slides per jar) for 5 minutes. Do not denature more than 6 slides at one time per Coplin jar. *Note: Verify the solution temperature before each use.*
- Using forceps, remove the slide(s) from the denaturing solution and immediately place into a 70% ethanol wash solution at room temperature. Agitate the slide to remove the formamide. Allow the slide(s) to stand in the ethanol wash for 1 minute.
- Remove the slide(s) from 70% ethanol. Repeat step 5 with 85% ethanol, followed by 100% ethanol.
- Drain the excess ethanol from the slide by touching the bottom edge of the slide to a blotter, and wipe the underside of the slide dry with a laboratory wipe.
- Dry the slide(s) on a 45-50°C slide warmer for 2-5 minutes.

b. Probe Preparation

- Allow the probe to warm to room temperature so that the viscosity decreases sufficiently to allow accurate pipetting.
- Vortex to mix. Centrifuge each tube for 2-3 seconds in a bench-top microcentrifuge to bring the contents to the bottom of the tube. Gently vortex again to mix.

c. Hybridization

- Apply 10 µL of probe mixture to target area of slide. lmnxdiately, place a 22 mm x 22 mm glass coverslip over the probe and allow it to spread evenly under the coverslip. Air bubbles will interfere with hybridization and should be avoided. The remaining probe solution should be refrozen immediately after use.
- Seal coverslip with rubber cement as follows: Draw the rubber cement into a 5 mL syringe. Eject a small amount of rubber cement around the periphery of the coverslip overlapping the coverslip and the slide, thereby forming a seal around the coverslip.
- Place slides in the pre-warmed humidified hybridization chamber. Cover the chamber with a tight lid and incubate at 37°C overnight (14-18 hours).

d. Post-Hybridization Washes

- Add POST-hybridization wash buffer (2X SSC/0.3% NP-40) to a Coplin jar. Prewarm the post-hybridization wash buffer by placing the Coplin jar in the 72±1°C water bath for at least 30 minutes or until solution temperature has reached 72±1°C. Note: The temperature of the wash solution must return to 72°C before washing each batch.
- Add post-hybridization wash buffer to a second Coplin jar and place at room temperature. Discard both wash solutions after 1 day of use.
- Remove the rubber cement seal from the first slide by gently pulling up on the sealant with forceps.
- Immerse slide(s) in post-hybridization wash buffer at room temperature and float off coverslip.
- After coverslip has been carefully removed, remove excess liquid by wicking off the edge of the slide and immerse slide in post-hybridization wash buffer at 72±1°C for 2 minutes (≤6 slides/jar).
- Remove each slide from the wash bath and air dry in the dark in an upright position. (A closed drawer or a shelf inside a closed cabinet is sufficient.)

- Apply 10 µL of DAPI counterstain to the target area of the slide and apply a glass coverslip. Store the slide(s) in the dark prior to signal enumeration.

e. Slide Storage

- Store hybridized slides (with coverslips) at -20°C in the dark. After removing from -20°C storage, allow slide(s) to reach room temperature prior to viewing using fluorescence microscopy.

2. Signal Enumeration

a. Assessing Slide Adequacy
Evaluate slide adequacy using the following criteria:

- Probe Signal Intensity: The signal should be bright, distinct, and easily evaluable. Signals should be in either bright, compact, oval shapes or stringy. diffuse, oval shapes.
- Background: The background should appear dark or black and relatively free of fluorescence particles or haziness. If any of the above features are unsatisfactory, consult the trouble-shooting guide (Table 2) and process a fresh slide.

b. Recognition of Target Signals

Use the prescribed filter (DAPI/Orange/Green). Adjust the depth of the focus, and become familiar with the size and shape of the target signals and noise (debris). Enumerate hybridization signals only among tumor cells. Tumor cells in general are larger than normal cells, lymphocytes, and epithelial cells. Identify target areas by H & E stain . Identify these areas on the coverslip after the FISH assay is performed.

c. Selection of Optimum Viewing Area and Evaluable Nuclei

Use a 20X objective to view the hybridized area and locate the target of interest (tumor cells as identified by H & E stain). Avoid areas of necrosis and where the nuclear borders are ambiguous. Skip those nuclei with signals that require subjective judgment. Skip signals with weak intensity and non-specificity, or with noisy background. Skip nuclei with insufficient counterstain to determine the nuclear border. Enumerate only those nuclei with discrete signals.

d. Signal Enumeration

Using a 4OX objective, scan several areas of tumor cells, to account for possible heterogeneity. Select an area of good nuclei distribution; avoid areas of the target where hybridization signals are weak. Using a 63X or IOOX objective, begin analysis in the upper left quadrant of the selected area and, scanning from left to right, count the number of signals within the nuclear boundary of each evaluable interphase cell according to the guidelines provided below.

- Focus up and down to find all of the signals present in the nucleus.
- Count two signals that are the same size and separated by a distance equal or less than the diameter of the signal as one signal.
- Do not score nuclei with no signals or with signals of only one color. Score only those nuclei with one or more FISH signals of each color.
- Record counts.

Continue this process until 60 nuclei are enumerated and analyzed.

VI. PROCEDURE NOTES / METHOD PERFORMANCE SPECIFICATIONS.

A. INTERPRETATION OF RESULTS

1. The number of LSI HER-2/*neu* and CEP 17 signals per nucleus are recorded in a table. Results on enumeration of 60 interphase nuclei from tumor cells per target are reported as the ratio of average HER-2/*neu* copy number to that of CEP 17. Our clinical study found that specimens with amplification showed a LSI HER-2/neu:CEP 17 signal ratio of $\geq$2.0 and normal specimens showed a ratio of <2.0 (9).

2. Results at or near the cutoff point (1.8 - 2.2) should be interpreted with caution. The specimen slide should be re-enumerated by another technician to verify the results. If still in doubt, the assay should be repeated with a fresh specimen slide. If the test results are not consistent with the clinical findings, a consultation between the pathologist laboratory director and the treating physician is warranted.

B. REASONS TO REPEAT THE ASSAY

The following are situations requiring repeat assays with fresh specimen slides and the appropriate control slides. Consult the troubleshooting guide (Table 2) for probable causes and the actions needed to correct specific problems.

1. If the control slides fail to meet the slide acceptance criteria, the specimen slide results are not reliable, and the assay must be repeated.

2. If there are fewer than 60 evaluable nuclei. The test is uninformative and the assay should be repeated.

3. If, upon assessing the slide quality as described in the Signal Enumeration section, any of the aspects (signal intensity, background, or cross-hybridizadon) are unsatisfactory, the assay must be repeated.

C. LIMITATIONS

1 . The PathVysion Kit has been optimized only for identifying and quantifying chromosome 17 and the HER-2/*neu* gene in interphase nuclei from formalin-fixed, paraffin-embedded human breast tissue specimens. Other types of specimens or fixatives should be optimized separately.

2. The clinical interpretation of any test results should be evaluated within the context of the patient's medical history and other diagnostic laboratory test results.

3. FISH assay results may not be informative if the specimen quality and/or specimen slide preparation is inadequate.

4. Technologists performing the FISH signal enumeration must be capable of visually distinguishing between the orange and green signals.

VII. QUALITY CONTROL

A. QUALITY OF SLIDES.

1. Check all slides grossly and microscopically for substandard staining or mounting. This should include inspection for the following:
   a. All numbers within each case are consistent.
   b. All numbers and letters are legible.
   c. No bubbles are retained in the mounting medium.
   d. No "floaters" are present.
   e. No smudged or messy staining.

2. All deficiencies will be corrected as follows:
   a. Cutting, labelling and floaters will be returned to microtomist.
   b. Bubbles and coverslipping errors will be corrected by technician assembling slides.
   c. Poor hematoxylin & eosin staining will be returned to technician running stainer.

B. QUALITY CONTROL OF HYBRIDIZATION.

1. Internal control benign and normal cells should be inspected to confirm that the nuclei of these cells contain the normal number of HER-2/neu signals (2) and the normal number of chromosome 17 centromeres.

2. An amplification control, for example SK-BR-3 human breast cancer cells, should be included with procedure.

3. The quality of all immunostained slides will be assessed by microscopic review of laboratory director. Any inadequately prepared slides will require that the procedure be repeated.

4. If control slides fail to meet the slide acceptance criteria, the assay may not have been performed properly or the PathVysion Kit component(s) may have performed inadequately. A repeat analysis with fresh control slides and patient specimen slide(s) will be necessary.

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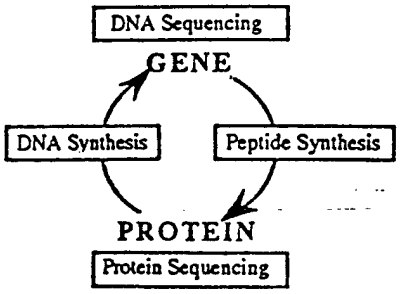

DNA Sequencing Protocol

Introduction

Our automated DNA sequencing method is a dye-based (usually BigDye™), Applied Biosystems procedure, where 3'-fluorescent-labeled dideoxynucleotide triphosphates (dye terminators) are incorporated into DNA extension products (asymmetric PCR). During the electrophoresis process, the fluorescent labels are excited by laser-light and the resulting specific emissions are detected and analyzed by computer. The collected data are then stored on a computer disk or printed. The results can then can be further processed, (e.g., translated or aligned with other templates) using sequence-editing programs. Data will be stored by the Core Laboratory for no more than two months.

Both plasmid and PCR products can be sequenced using the above procedure. Depending upon base composition of the sample, different preparation and sequencing strategies can be employed to optimize the results. Strategies for sequencing difficult templates should be discussed on an individual basis.

To assist in the preparation of the template, a number of standard and commercially available purification procedures are listed below. A section on primer design and preparation is also included.

Sample Delivery

A Sequencing Request Form must accompany each sequencing order. Samples should be submitted in labeled 500µl microtubes. The sample titles, written on the tops of the tubes, should match those listed on the request form. The sample titles should not be more than six characters long. The sample tubes should not be parafilmed. If you anticipate sequencing difficulties due to template structure, please discuss your concerns prior to sample submission. Comments can also be added in the appropriate section of the Sequencing Request Form.

The template and single primer must mixed and suspended in a total volume of 18 µl sterile deionized water. The samples should be maintained at -20°C for storage and transportation.

Required quantities of primers and templates are follows:

- 4.8 pmoles of primer
- 0.75 µg of plasmid DNA or 0.15 µg of PCR product.

DO NOT DEVIATE FROM THESE REQUESTED QUANTITIES.

Cell Lines

Host cell strains can sometimes impact template quality. The presence of large amounts of carbohydrates and high levels of endonuclease activity within certain strains can lower the quality and quantity of DNA isolated.

DH5α: Highly Recommended
MV1190 and XL1 Blue: Recommended - Some variability noticed
JM101: Not Recommended

Sample Purification

Since a small amount of DNA is needed for sequencing, one should never sacrifice quality for quantity. Always quantitate your final product spectrophotometrically. Do not overload your purification system. Remember that selection markers have variable degradation rates depending on the culture environment.

Even though DNA obtained from minipreps can yield good sequencing results, they are not as forgiving as large-scale preps. Using small 6 to 8 hour growing bacterial cultures or dividing an isolated colony between two minipreps and combining the products, will circumvent problems associated with some miniprep systems.

The purity of DNA should be verified on an agarose gel until the purification process can be confidently performed. PCR products and linearized plasmids should run as a single sharp band on the agarose gel. Smears or alternate products within the sample lanes are prognostic of poor sequencing results. Such samples should be further purified from solution or by gel purification.

Plasmid Purification:

(1) Cesium Chloride Banding
Refer to Current Protocols in Molecular Biology (Wiley) or equivalent. Exposing an aliquot of DNA to UV light helps to verify that ethidium bromide has been removed. Precipitating DNA a second time ensures complete removal of salts.

(2) QIAGEN plasmid prep systems (various scales) (QIAGEN Inc.)

(3) UltraClean™ Miniprep or equivalent quality larger scale (McFrugal's Lab Depot)

(4) PERFECTprep™ or equivalent quality larger scale (5 Prime —» 3 Prime)

PCR Product Purification from Solution:

(1) Microcon™ Microconcentrators (various MWCO) (Amicon® Inc.)

(2) QIAquick PCR Purification Kit (QIAGEN Inc.)
PCR Products > 100bp

PCR Product Purification from Agarose Gels:

Use low melting temperature agarose and TAE as running buffer. Purify the DNA using preferably one of the following kits. Elute the DNA with sterile deionized water.

(1) QIAEX® II Gel Purification Kit (QIAGEN Inc.)
PCR Products > 40bp

(2) GENECLEAN (BIO 101 Inc.)
Products > 200bp

(3) MERmaid Kit™ (BIO 101 Inc.)
Products 10 to 200bp

(4) Micropure™ Separator combined with a Microcon™ Microconcentrator (Amicon® Inc.)

Primer Design and Quantitation:

Nested primers or primers used for the original PCR can be used for sequencing. The following criteria should be considered when designing sequencing primers:

-18 to 24 bases long
-GC content: 50-55%
-Tm > 50°C (preferably 55-60°C)
-Repetition of any single base should be less than four
-Possible primer-dimer formation should be avoided

To quantitate primer concentration, use the following formula:

$(A260 \times 100) / (1.54nA + 0.75nC + 1.17nG + 0.92nT)$ = pmole/µl of diluted sample

Multiply by the dilution factor to obtain the stock concentration.

Rm. NOR 5338 1441 Eastlake Ave Los Angeles, CA 90089-9176 Phone (323)865-0541 FAX (323)865-0091